UNIVERSITY OF ALBERTA

TOLERANCE INDUCTION IN EXPERIMENTAL ISLET TRANSPLANTATION

BY

©

SULAIMAN NANJI
BSc, MD

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DEDICATION

This thesis is dedicated to my wife, Farah, who has inspired me throughout this endeavour, and from whom I have learned to appreciate that any journey is as spectacular as the destination, not only for the intellect but also for the heart and spirit.

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LIST OF ABBREVIATIONS

ALS	anti-lymphocyte serum
APC	antigen presenting cell
B7RP-1	B7-related protein-1
BMT	bone marrow transplant
CD40L	CD40 ligand
CFSE	carboxyfluorescein diacetate succinimidyl ester
CsA	cyclosporine
CTLA4	cytotoxic-T-lymphocyte-associated Protein 4
CTX	cyclophosphamide
DMEM	dulbecco's modified eagle's medium
DST	donor-specific transfusion
FCS	fetal calf serum
GVHD	graft-versus-host disease
HBSS	Hank's balanced salt solution
IBMIR	instant blood mediated inflammatory reaction
ICOS	inducible costimulatory molecule
IL	interleukin
mAb	monoclonal antibody
MHC	major histocompatability complex
mTOR	molecular target of rapamycin
NFAT	nuclear factor of activated T-cells
NOD	non obese diabetic
PCR	polymerase chain reaction
PD-1	programmed death-1
PERV	pig endogenous retrovirus
PFC	perfluorocarbons
PTLD	post transplant lymphoproliferative disorder
STZ	streptozotocin
TBI	total body irradiation
TCR	T cell receptor
TGF-β	transforming growth factor-beta
T_{REGS}	regulatory T cells
TNF	tumor necrosis factor
WBC	white blood cell

CHAPTER ONE

THE PATH TO TRANSPLANTATION TOLERANCE: GENERAL INTRODUCTION AND RATIONALE

1.1 A PLEA FOR TRANSPLANTATION TOLERANCE

The origins of whole organ transplantation can be dated back to Alexis Carrel's pioneering work in Experimental Surgery (Figure 1-1). Once he had developed techniques for end-to-end anastamoses of blood vessels, he wanted to determine if a transplanted organ could function normally in a recipient of the same species. In 1908, Dr. Carrel transplanted kidneys between cats and found that some of the recipients maintained urinary output for up to 25 days after transplantation. Although all the cats eventually died, his experiment was a success insofar as it confirmed that a transplanted organ could carry out its normal function in a recipient.

Figure 1-1: Alexis Carrel (1873-1944)
Recipient of the Nobel Prize in Physiology or Medicine in 1912
for his work on vascular anastomoses.

In 1933, the first human kidney transplant was attempted by a Russian surgeon named Voronoy. Using local anesthesia, the kidney was transplanted into a subcutaneous pouch in the right groin of the recipient. The ureter was exteriorized from the pouch and vascular anastomoses were created onto the femoral vessels according to the techniques originally described by Carrel (Figure 1-2). Due to a mismatch in blood types between the donor and the recipient, hyperacute rejection of the kidney ensued and the patient subsequently died before renal function could be established.

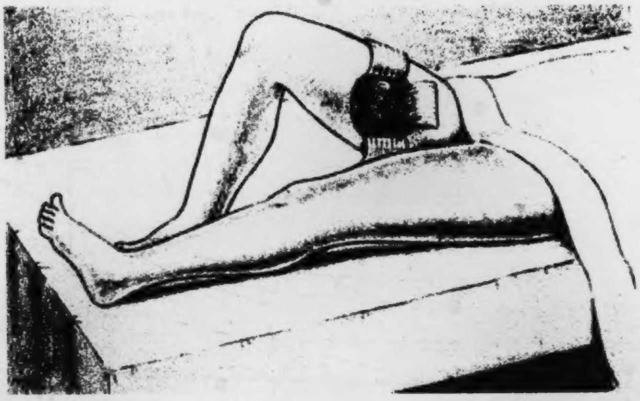

Figure 1-2: Technique used by Voronoy for the first human renal transplant (1933).
(From An Illustrated History of Organ Transplantation, Kuss & Bourget. 1992, p.36)

In 1954 Joseph Murray accomplished the first successful human kidney transplant between two 23 year old identical twins at the Brigham hospital in Boston (Figure 1-3). The recipient was completely cured of his glomerulonephritis, returned to work, and went on to lead a normal life with his family. This first case of successful human transplantation generated incredible enthusiasm and prompted keen interest in clinical organ transplantation. This year marks the 50th anniversary of this historic achievement, and through tremendous efforts over the past half a century, transplantation has now become the life-saving therapy of choice for individuals suffering from end-stage organ failure.

Figure 1-3: First successful human transplantation between two Identical twins in 1954 by the Murray, Merrill and Harrison team. (From An Illustrated History of Organ Transplantation, Kuss & Bourget. 1992, p.45)

Today, liver, pancreas, kidney, heart, lung, small bowel, bone marrow, islet and cornea transplants are performed among non-identical individuals with increasing frequency and success worldwide. While improvements in surgical techniques have essentially eliminated one substantial barrier in clinical transplantation, other significant challenges remain that continue to confine the full therapeutic potential of organ transplantation. Although one ever-present challenge is the critical shortage of donor organs for the increasing numbers of recipients, another formidable obstacle to successful transplantation is the immune system. Since almost all transplants involve genetically dissimilar donor-host combinations (allografts), it is imperative that the recipient receives potent anti-rejection drugs in order to prevent the body's immune system in initiating its natural defense against foreign tissue. Without suppression of the immune system the allograft would be destroyed in the same way our immune system destroys harmful pathogens that are foreign to our body.

During the past several years, considerable advances have been made in the development of more potent and less toxic immunosuppressive agents (Figure 1-4). The pharmacologic armamentarium available to the transplant physician today offers a remarkable challenge in discerning the most effective and risk-adapted treatment combinations, and this is becoming increasingly more perplexing as newer agents are being developed. Nonetheless, the clinical application of these newer agents has significantly improved short-term graft survival times resulting in superior patient outcomes (1). These agents have also played an important role in making clinical islet transplantation a safe and effective therapy for select patients with type I diabetes, when

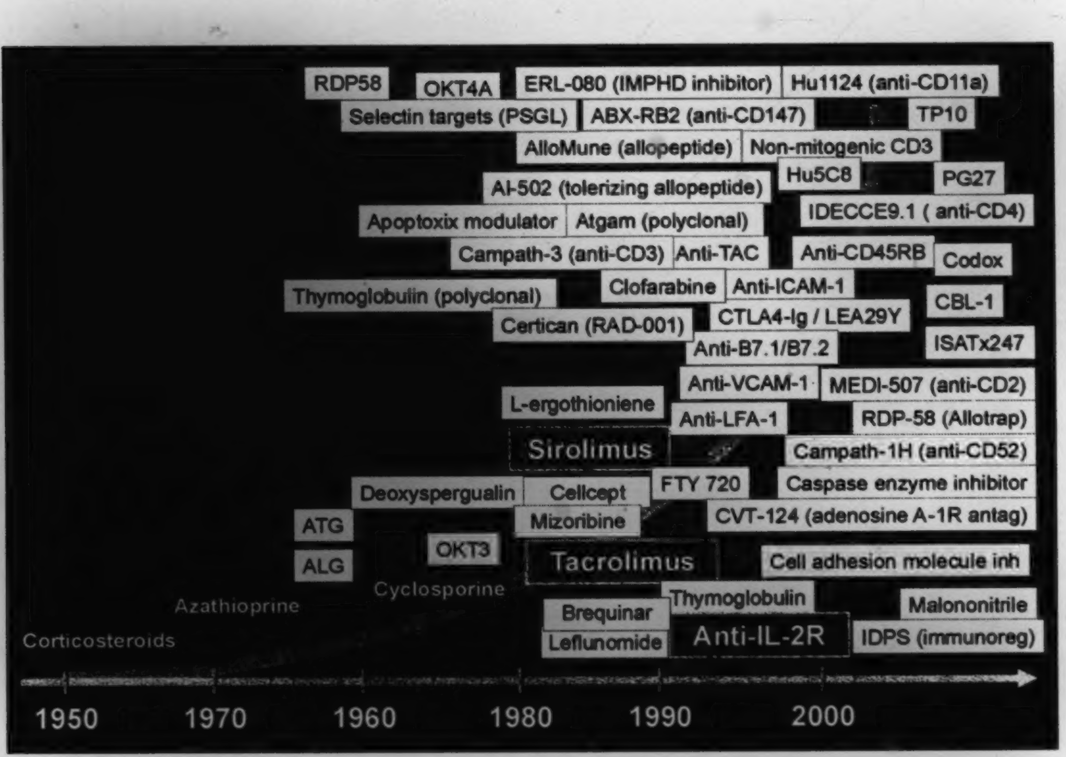
RDP58
OKT4A
ERL-080 (IMPHD inhibitor)
Hu1124 (anti-CD11a)
Selectin targets (PSGL)
ABX-RB2 (anti-CD147)
TP10
AlloMune (allopeptide)
Non-mitogenic CD3
Hu5C8
PG27
AI-502 (tolerizing allopeptide)
IDECCE9.1 (anti-CD4)
Apoptoxix modulator
Atgam (polyclonal)
Campath-3 (anti-CD3)
Anti-TAC
Anti-CD45RB
Codox
Thymoglobulin (polyclonal)
Clofarabine
Anti-ICAM-1
CBL-1
Certican (RAD-001)
CTLA4-Ig / LEA29Y
Anti-B7.1/B7.2
ISATx247
Anti-VCAM-1
MEDI-507 (anti-CD2)
L-ergothioniene
Anti-LFA-1
RDP-58 (Allotrap)
Sirolimus
Campath-1H (anti-CD52)
FTY 720
Caspase enzyme inhibitor
Deoxyspergualin
Cellcept
ATG
Mizoribine
CVT-124 (adenosine A-1R antag)
OKT3
ALG
Tacrolimus
Cell adhesion molecule inh
Cyclosporine
Azathioprine
Thymoglobulin
Malononitrile
Brequinar
Corticosteroids
Leflunomide
Anti-IL-2R
IDPS (immunoreg)
1950
1970
1960
1980
1990
2000

Figure 1-4: Evolution of more specific and less toxic immunosuppressive anti-rejection therapies in clinical trials.
In the early era of clinical islet transplantation mainstay anti-rejection therapy included corticosteroids, azathioprine, and cyclosporine. Immunosuppression in the Edmonton Protocol consists of sirolimus, tacrolimus, and anti-IL-2 receptor induction (Adapted form A.M.J. Shapiro, PhD Thesis 2001, with kind permission).

the success rates of previous attempts was only approximately 8% (2-4). Although advances in the field of immunosuppression have made an enormous contribution to the improved success in transplantation, the detrimental effects of these drugs remain pervasive. They are not always effective, must be taken daily for life, are expensive, and have potential toxicities such as renal dysfunction. Recently, the development of chronic renal failure after transplantation of nonrenal organs has been identified as a significant concern, with an associated four and a half-fold increase risk of death of the recipient (5). Furthermore, these drugs are associated with deleterious metabolic effects such as diabetes and hypercholesterolemia (1), which in turn lead to ischemic heart disease, the cause of death in approximately half of transplanted patients (6). Also, due to the non-specific nature of these agents, patients are at increased risk for malignancies such as lymphoma, as well as opportunistic infections, which can be severe and sometimes fatal. Moreover, although these agents have considerably improved short-term outcomes in transplanted patients, recipients are still at risk for eventually losing their graft due to chronic rejection (7).

Presently, immunosuppression is essential for the transplantation of organs between genetically dissimilar individuals. In accepting the allograft, the recipient must also accept the harmful effects associated with anti-rejection drugs. An attractive alternative to immunosuppression is to modify the recipient's immune system to specifically ignore or "tolerate" the donor tissue without altering its normal protective function against pathogens (8). This concept of immunologic tolerance was first described by experimental embryologists in the 1930's, and it was first experimentally induced in neonatal mice by Peter Medawar and his colleges in 1953 (9). Since then, remarkable efforts have been made to reliably induce a state of life-long, drug-free graft acceptance in a variety of animal models of transplantation. Through these experiments, we have learned that tolerance is not merely the absence of an immune response to donor antigen, rather it may involve a variety of mechanisms that actively regulate the immune response to specifically accept donor antigen, while maintaining host immunity to pathogens. The evolution of our understanding of immunologic pathways responsible for graft rejection and acceptance, together with the development of novel immunosuppressive drugs, has led to the development of innovative strategies for experimental tolerance induction. It has also led transplant immunologists to develop an operational definition of tolerance: The maintenance of normal allograft function in the absence of exogenous immunosuppression. Although there are countless reports of successful tolerance protocols in several animal models of transplantation, a significant leap must yet be made from the successful experimental protocols in these models to clinical reality.

The realization of practical tolerance inducing strategies for clinical transplantation would revolutionize this field completely. Patients undergoing organ transplantation would be able to maintain a functioning allograft for life without any of the morbidities associated with life-long immunosuppression. Eradicating the threat of acute and chronic rejection would ensure that only a single transplant would be required for an individual, thereby reducing the burden of donor organ shortage. Moreover, the development of clinical tolerance protocols may also provide considerable therapeutic benefit in other clinical contexts such as autoimmunity or allergy (10). Finally, the financial implications of eliminating the need for expensive anti-rejection drugs on the health-care industry would be astounding. Given the enormous potential benefits of clinical tolerance induction, it seems very appropriate that Peter Medewar referred to the establishment of tolerance as "the Holy Grail" of transplant immunology (Figure 1-5).

Figure 1-5: Sir Peter Medawar (1915-1987)
Recipient of the Nobel Prize in Physiology or Medicine in 1960 for his work in transplant immunology. (From An Illustrated History of Organ Transplantation, Kuss & Bourget. 1992 Laboratoires Sandoz, France)

This introductory chapter will provide an overview of our current understanding of immunologic tolerance in the setting of organ transplantation. First, a historical background will be provided to highlight particular individuals and discoveries that have had an enormous impact in the field of transplant immunobiology. This will be followed by a brief discussion of the biologic mechanisms involved in the alloimmune response, to impress that tolerance is not simply the absence of an immune response, but an active process involving various cellular interactions and mechanisms. Finally, key strategies that have proven promise in the induction of transplantation tolerance will be described.

1.2 HISTORICAL PERSPECTIVE

In the first half of the twentieth century, embryologists were profoundly intrigued with the study of factors influencing the differentiation of the embryo and its component parts. Some of the most challenging questions asked centered around the extent to which differentiation was already pre-programmed versus the influence of factors arising from local microenvironments. These and other questions were investigated primarily through studies in vitro, but another increasingly popular approach involved the transplantation of embryonic tissues. At this time, based on earlier studies, researchers were already aware that transplantation of tissues between genetically dissimilar adults was never successful. Consequently, the transplantation of embryonic amphibian tissues into amphibian embryos or larvae or, more commonly, embryonic chick tissues to chick embryos was employed with remarkable success. Subsequently, experimental embryologists attempted transplanting xenogenic tissues to chick embryos, which was also very successful. As a result, transplantation of embryonic tissue became a widely used method for the study of embryonic differentiation. Embryologists now had a powerful tool that allowed them to delve further into the intricacies of differentiation, however, none gave any consideration as to the reasons why transplants were successful in chick and amphibian embryos but destined for failure in adult animals. The closest embryologists got to understanding the basis of their successful transplants came about in the late 1930's when an embryologist by the name of Eastlick studied the transplantation of embryonic tissues of other avian species to the chick embryo. Many of his grafts survived and differentiated, however, some demonstrated "incompatability reactions" close to or after hatching, which he attributed to the presence of foreign proteins in the graft that "sensitize the hosts so that the defense mechanisms are brought into play." He also commented that in the embryo the graft and host tissues "may have become more or less "adjusted" to one another during the embryonic period, and that this tolerance may not be lost at once" (11). This was the first time that the term tolerance had been applied.

The concept of chimerism was conceived in 1945 when R.D. Owen, an Associate Professor of Genetics and Zoology at the University of Wisconsin, described a naturally occurring state of mixed chimerism in cattle. He discovered that dizygotic bovine twins that shared a common placental circulation demonstrated "a mixture of two distinct types of erythrocytes" long after birth (12). Owen speculated that this state of red cell chimerism was the result of the exchange of hematopoietic stem cells during embryonic life via a shared placenta. A few years after this discovery, Peter Medawar, a Professor of Zoology at Birmingham University, and his colleges provided experimental evidence for Owen's theory through a series a skin grafting experiments in cattle. Interestingly, the idea to pursue these experiments emerged as a result of a chance meeting between Medawar and a Scottish geneticist named H.P. Donald. Donald was studying genetic and environmental differences in cattle using identical and fraternal twins. A key problem he faced in his study was the inability to distinguish between the two kinds of twins. During their fortuitous meeting over cocktails, Medawar suggested to Donald that his problem could be solved by simply exchanging skin grafts between the twins and observing how long they last. If the graft lasts indefinitely then the twins are identical; conversely, if the graft fails after a week or two then the twins are certainly fraternal. This conversation led to a joint venture between the Medawar and Donald during which Medawar and his post-doctoral fellow Rupert Billingham mastered the technique of skin grafting in calves. The results of their experiments were completely unanticipated. Both identical and fraternal twins, including twins of opposite sex, accepted their grafts. However, they rejected grafts from their parents or from siblings of separate birth, as predicted. In their publication of these results in 1951 (13), they indicated that their findings provided experimental evidence for Owen's hypothesis that stem cells precursors must be exchanged between twins during embryonic development.

Shortly thereafter, in 1952, Cannon and Longmire conducted a series of experiments investigating the acceptance of skin allografts transplanted in chicks soon after hatching. Although the majority of the skin allografts were rejected, they discovered that a few allografts actually survived far longer than anticipated. Upon closer observation, they realized that this prolonged survival occurred in chicks transplanted within the first 3 days of hatching, and that this survival could be extended by treating the chicks with cortisone (14).

A year later, Medawar and colleagues provided an explanation for Cannon and Longmire's observations by the phenomenon of acquired immunologic tolerance (9). Since Medawar's observations with the cattle skin graft experiment, he began working towards a strategy to experimentally induce tolerance. His experimental approach involved the inoculation of mouse embryos and neonatal mice with a suspension of allogeneic hematopoietic cells. Mice having survived this procedure were given a skin graft from the same donor strain that supplied the foreign cells for the inoculation. Interestingly, the majority of grafts demonstrated either prolonged or permanent acceptance. Moreover, Medawar demonstrated that tolerant mice accepted a second skin graft from the donor but not a graft from an unrelated third party strain, thereby indicating the specificity of tolerance. Furthermore, he showed that tolerant grafts that were re-transplanted to mice syngeneic with the host animals were rejected. From this latter observation, he concluded that tolerance was due to the specific failure of the host's immunologic response as opposed to graft adaptation. The publication of these results (9) represented the first demonstration ever of experimentally induced tolerance and provoked quite an arousal in the biologic community.

Around the same time as Medawar's observations, Milan Hasek in Prague demonstrated that parabiosis of different strain chick embryos induced an immune hyporesponsive state to each other's red cells, further supporting the notion that exposure to foreign antigen during the development of the immune system leads to tolerance rather than heightened immunity (15).

During the subsequent years following these landmark publications, Medawar's group continued to explore the concept of tolerance. Their experiments culminated in another historical publication in 1956, in which they established the immunologic basis of tolerance (16). This paper confirmed the specificity of tolerance and also demonstrated that tolerance was not an all or nothing phenomenon, but that it involved varying degrees of graft acceptance from transient to permanent. They also demonstrated that a variety of nucleated cells, but not red cells, could induce it.

At this point in time, the induction of tolerance to transplanted grafts could only be achieved in neonatal animals. This prompted Brent and Gowland to probe the question of immunologic immaturity, and to determine what factors influenced the induction of tolerance in an immature immune system. In their series of experiments they demonstrated that the emerging immune system of young mice could be altered to induce tolerance through the administration of large doses of allogeneic cells over a prolonged period of time. From this and other important observations, they concluded that the difficulties in establishing tolerance in post-natal life are due to the emergence of increasing numbers of immunologically competent cells from birth onwards (17, 18).

The concept of neonatally acquired transplant tolerance was first suggested by Macfarlane Burnet, an Australian virologist and physician who proposed the famous "self-nonself" hypothesis for immune development. Burnet recognized a similarity between Owen's red cell chimeric model in the dizygotic cattle twins and the phenomenon induced by inoculation of foreign embryonic cells in the chick embryo, and hypothesized that tolerance could be acquired by fetal exposure to nonself constituents. Medawar's studies involving skin grafting experiments between dizygotic cattle twins and subsequent evidence that tolerance could be achieved in mice by inoculation of embryos or newborn mice with allogeneic cells verified Burnet's hypothesis. For their work in transplant immunology, Medawar and Burnet were awarded the Nobel Prize in 1960 in Physiology or Medicine. This work formed the basis of the long-standing, prevalent view that tolerance is an intrinsic property of the newly developing immune system. However, in recent years, this notion has been challenged by several landmark reports demonstrating that newborn mice are in fact capable of responding to antigen, depending on the dose (19), adjuvant (20), and nature of the APC (21).

While Medawar and his colleagues were the first to ever experimentally induce tolerance and subsequently explain the immunologic basis of tolerance, they had no concept of the clinical relevance of their findings. Interestingly, after a lecture given by Medawar in 1957, he was asked by Roy Calne whether he thought that the phenomenon of tolerance had any clinical application, to which he replied "Absolutely none" (22). At that time his response was very appropriate based on what was understood about tolerance then. Namely, that is was highly specific and that it depended upon the age of the animal. Given the relative maturity of the immune system in human neonates, most researchers believed that tolerance induction in adult animals was not possible and, therefore, had no clinical relevance (23). Today, however, almost 50 years after Medawar's monumental discoveries, the clinical relevance of tolerance induction cannot be overemphasized.

1.3 TRANSPLANTATION IMMUNOLOGY

The following section is brief overview of the relevant mechanisms involved in the allo-immune response, with particular emphasis on the two-signal model of T cell activation. The mechanisms of tolerance are discussed and the role of regulatory T cells is highlighted.

1.3.1 THE ALLO-IMMUNE RESPONSE

The T lymphocyte is the principal player in directing the immune response against an allograft. During the complex process of alloimmunity, recipient T cells experience many different immunological signals, which influence their response towards either allograft rejection or acceptance. The myriad of factors involved in dictating the fate of T cells are still not well delineated, and how these factors interact to influence T cells to undergo immunologic responses versus tolerogenic responses remains a matter of much speculation. Ultimately, however, it is the collective contribution of individual T cell responses that determine the fate of the graft.

The immune response involved in the rejection of an allograft consists of two distinct pathways, the **direct and indirect pathways of antigen recognition** (Figure 1-6). The direct pathway is exclusive to the alloresponse while the indirect pathway resembles the conventional form of antigen presentation (24). In the direct route, recipient allospecific T cells recognize intact donor-MHC peptide complexes on donor antigen-presenting cells (APCs). In the indirect pathway, host APCs process donor-derived protein into peptides, and these peptides are then presented in the context of host MHC molecules to the recipient's T cells (25). The direct pathway represents the initial immediate encounter of recipient T cells with donor antigen. It is believed that this pathway is eventually extinguished due to the diminishing amount of donor APCs necessary to drive this pathway (8). Conversely, the indirect pathway can function indefinitely provided there are sufficient existing donor cells that can be processed by host APCs. It is postulated that the direct pathway has greater importance for acute rejection, whereas the indirect pathway is crucial to the progression of chronic rejection (26, 27). Nevertheless, the host must become tolerant to both pathways if the induction of transplantation tolerance is to be successful (28).

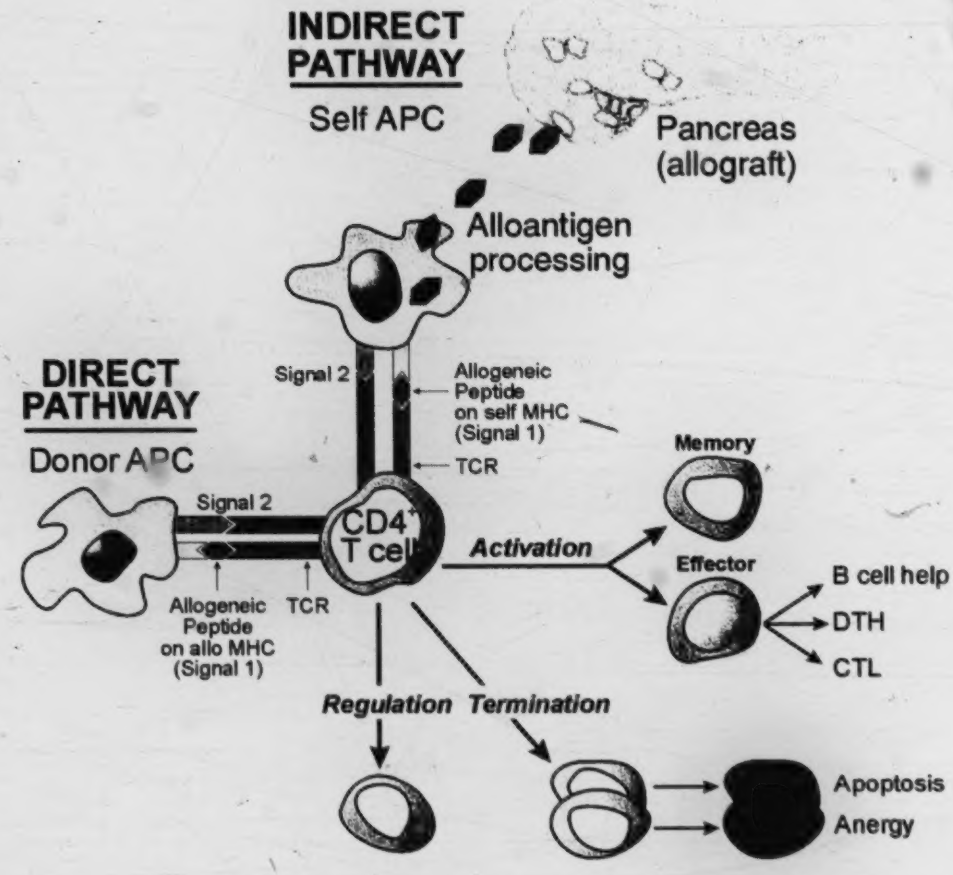
INDIRECT PATHWAY
Self APC
Pancreas (allograft)
Alloantigen processing
Signal 2
Allogeneic Peptide on self MHC (Signal 1)
TCR
DIRECT PATHWAY
Donor APC
Signal 2
CD4+ T cell
Allogeneic Peptide on allo MHC (Signal 1)
TCR
Memory
Activation
Effector
B cell help
DTH
CTL
Regulation
Termination
Apoptosis
Anergy

Figure 1-6: Direct and indirect pathways of allogeneic antigen recognition in graft rejection

1.3.2 THE TWO SIGNAL MODEL FOR T CELL ACTIVATION

In the mid-1970's, Lafferty and Cunningham expanded on earlier work by Bretscher and Cohn in developing the 2-signal model of T cell activation (29). This model proposes that in order to generate a productive immune response, 2 distinct but synergistic signals are required (30). The first signal is provided through the binding of the T cell receptor (TCR) with the MHC-peptide complex. This so-called "signal-one" is important in conferring antigen specificity to the immune response, but is unable to fully activate naïve T cells without "signal-two". This second signal, known as costimulation, is delivered by interactions between specific costimulatory molecules on the surface of T cells with their respective ligands on the APC. Costimulatory signals are not antigen-specific, rather, they synergize with TCR ligation, thereby allowing the T cell to produce the required cytokines for maintaining a sustained immune response (25) (Figure 1-7).

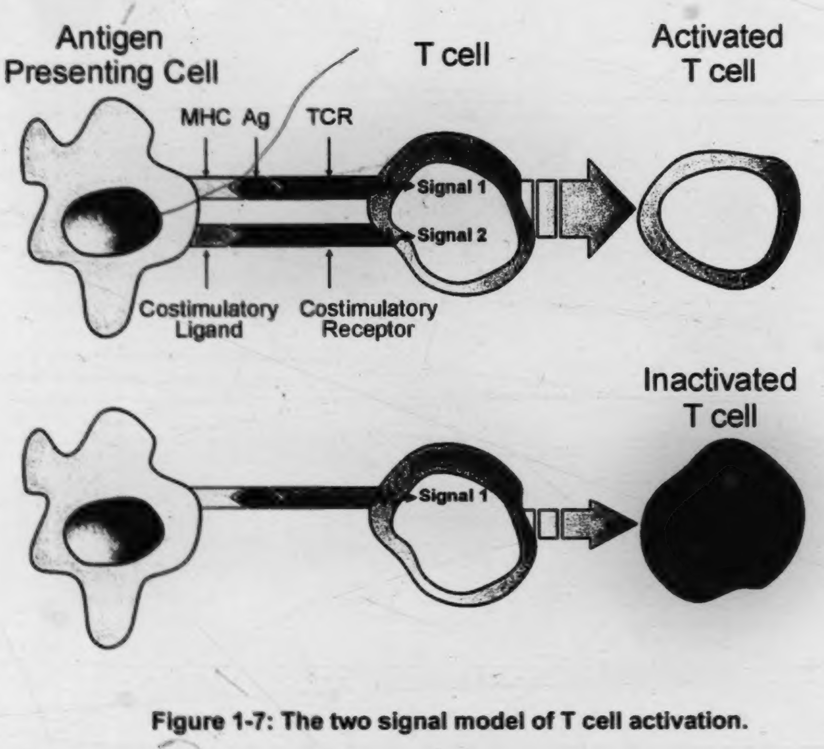
Antigen Presenting Cell
T cell
Activated T cell
MHC
Ag
TCR
Signal 1
Signal 2
Costimulatory Ligand
Costimulatory Receptor
Inactivated T cell
Signal 1

Figure 1-7: The two signal model of T cell activation.

Two very important corollaries arose from the 2-signal model of T cell activation (31). First, the delivery of signal 2 without recognition of the antigen-MHC complex by the TCR would be uneventful. Second, if the TCR appropriately recognized the antigen-MHC complex, but did not receive the costimulatory signal, then it would not be capable of producing an immune response. The second corollary suggested a novel approach to the induction of transplantation tolerance. That is, if signal 2 could be specifically interrupted at the time allo-reactive T cells encountered the transplanted graft, then they could be rendered inactive and unable to mount an immune response. Indeed, it has been shown that if a T cell recognizes antigen through its TCR without adequate costimulation, it can become anergic and undergo apoptosis (32-34).

In recent years, further investigation into T cell activation suggests that some of the cytokines produced during the T cell-APC interaction may provide an essential third signal that is required to fully activate T cells. This has been studied largely for CD8 T cells where IL-12 has been demonstrated to provide a third signal (signal 3) needed for strong proliferative and cytolytic responses. Therefore, with respect to naïve CD8 T cells, a three signal model has been proposed, where antigen (signal 1), costimulation (signal 2) and IL-12 and/or an alternate signal 3 is required for optimal activation (35-39).

1.3.3 COSTIMULATORY MOLECULES

There are several T cell molecules that provide costimulatory signals for T cell activation (Figure 1-8). One of the best characterized costimulatory receptors is CD28, which was discovered by June and colleagues in the mid-1980's (40). They found that stimulating T cells with a mixture of two antibodies, one that stimulated the TCR and another that bound to a separate cell surface receptor, resulted in rapid T cell proliferation. The cell surface receptor identified by that antibody was CD28. CD28 has two ligands, B7-1 (CD80) and B7-2 (CD86), both of which are expressed on activated APCs (33). Ligation of CD28 optimizes T cell responses through two general mechanisms. First, it induces T cells to respond at low levels of TCR ligation, thereby evoking an immune response even at low antigen concentrations (33). Second, CD28 costimulation sustains T cell activation, thereby preventing the induction of anergy or apoptosis seen with TCR binding alone (41, 42).

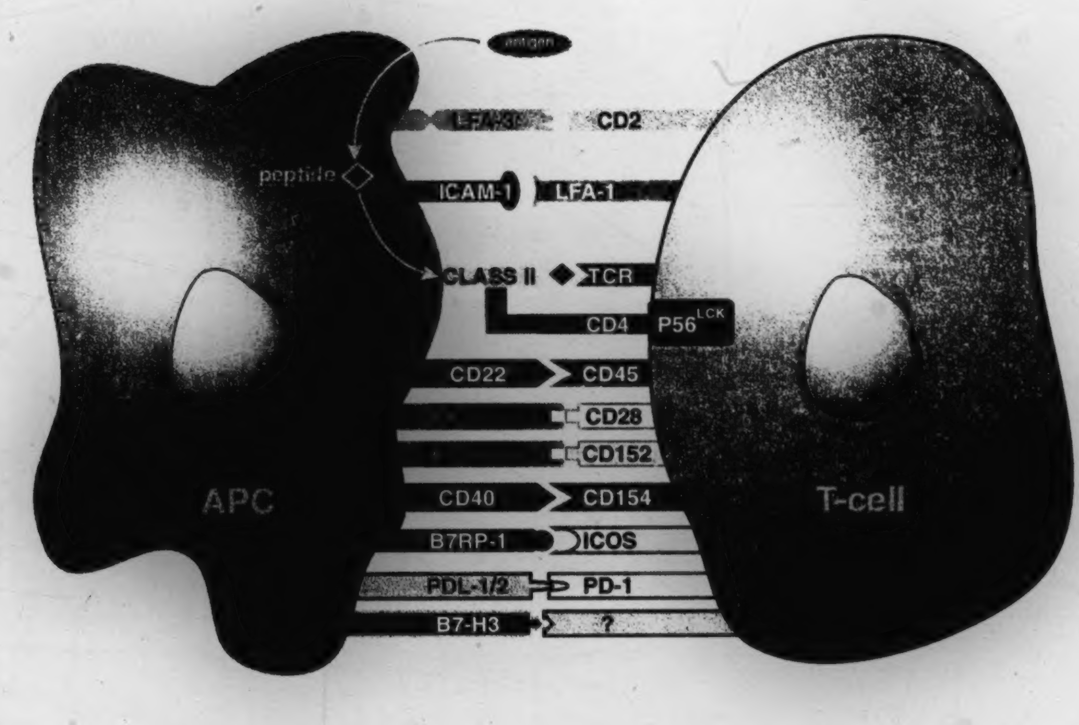
antigen
LFA-3
CD2
peptide
ICAM-1
LFA-1
CLASS II
TCR
CD4
P56LCK
CD22
CD45
CD28
CD152
CD40
CD154
B7RP-1
ICOS
PDL-1/2
PD-1
B7-H3
?
APC
T-cell

Figure 1-8: Costimulatory and coinhibitory molecules on the surface of the T cell. Regulation of immune responses to alloantigen is mediated through interactions between T cell associated molecules and their respective ligands on antigen presenting cells.

T cells also express another receptor called cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4 or CD152), which is structurally similar to CD28 and also binds B7-1 and B7-2, but with a higher affinity (41). The function of CTLA-4 was debated extensively until it was shown that CTLA-4 knock-out mice died prematurely from massive lymphocyte activation resulting in lymphocytic infiltration of the heart, pancreas, and other parenchymal tissues (43). Therefore, unlike CD28, CTLA-4 appears to transmit an inhibitory signal that acts to down-regulate the immune response (44). Since both CD28 and CTLA-4 share the same binding molecules on APCs, the latter acts as a competitive inhibitor to CD28-B7 costimulation in order to induce T cell anergy.

Another well described costimulatory pathway for T cell activation is the CD40-CD154 receptor pair. Although CD40 was originally described as a surface molecule on B lymphocytes important in mediating immunoglobulin class-switching (45), it has also been shown to exist on other APCs such as macrophages and dendritic cells (46). Binding of CD40 with its ligand CD154 (or CD40 ligand), not only triggers antibody production in B lymphocytes, but also induces B7 expression on all APCs (47), thereby promoting T cell activation through CD28-B7 costimulation. Moreover, stimulation of CD40 induces APCs to express adhesion molecules and inflammatory cytokines that are also important in T cell activation (48, 49).

More recently, a third member of the CD28/CTLA-4 family has been identified and termed *inducible* costimulatory molecule (ICOS) (50). Unlike CD28, which is constitutively expressed on all naïve T cells, ICOS is expressed by activated T cells and is retained on many memory T cells (51, 52). Despite its structural similarity with CD28, ICOS does not bind to the ligands B7-1 and B7-2, but with a novel molecule in the B7 family, namely B7-related protein-1 (B7RP-1) (53, 54). B7RP-1 is expressed on B cells, macrophages and dendritic cells, and ligation of this receptor with ICOS results in proliferation and differentiation of T cells (54-56). Further evidence of the importance of this costimulatory pathway in immune responses is derived from studies involving ICOS knock-out mice that demonstrate defective T cell activation and proliferation, as well as profound deficits in immunoglobulin isotype class switching (51, 57). Interestingly, however, class-switching was restored in these mice by CD40 stimulation, suggesting that ICOS facilitates collaboration between T cell and B cells through the CD40-CD154 pathway (57).

In comparison to the CD28:B7 and CD154:CD40 costimulatory pathways, the ICOS:B7RP-1 pathway has some unique characteristics. While CD28 and CD154 play a vital role in primary T cell activation, these molecules are significantly less important in the activation and maintenance of memory and effector T cell functions (58, 59). In contrast, although ICOS supports activation of naïve T cells, its role in the regulation of memory and effector T cell functions may be far greater (50, 51). Furthermore, while the blockade of the CD28:B7 and/or CD154:CD40 costimulatory pathways leads to prolonged allograft survival in numerous transplant models, it is not effective in preventing eventual graft loss due to chronic rejection (60, 61). However, the combination of CD154:CD40 costimulatory blockade with a specific monoclonal antibody (mAb) to block ICOS signaling has been effective in preventing chronic rejection in a murine model of cardiac transplantation (62).

In addition to ICOS, a fourth member of the CD28 family has been identified and named programmed death 1 (PD-1). Although originally described as a costimulatory molecule facilitating T cell proliferation (63, 64), further investigation has clearly demonstrated that this molecule inhibits cytokine production and T cell proliferation (65). The expression of PD-1 can be induced not only on T cells, but also B cells, myeloid cells and peripheral organs, suggestion that its role in regulating immune responses may be quite widespread, including the maintenance of peripheral tolerance (66). PD-1 has two recognized ligands termed PDL-1 and PDL-2, which are expressed on non-lymphoid tissues as well as APCs (67). Engagement of these ligands with PD-1 delivers a negative signal that likely acts synergistically with CTLA4 to terminate T cell responses. The importance of lymphocyte suppression by PD-1 is supported by observations of mice deficient in this molecule that suffer from autoimmune disorders due to inappropriate activation of T and B cells (68). Interestingly, although CTLA4 deficient mice typically die within 3-4 weeks, PD-1 deficient mice can survive for up to one year, suggesting that PD-1 is not the primary inhibitory signal for T cells.

The newest member of the B7 ligand family is B7-H3, with an as yet undefined CD28-like receptor. Although it is structurally homologous to other members of the B7 family, it is most closely related to the ICOS ligand B7RP-1 (69). B7-H3 has been found to be expressed on both lymphoid and non-lymphoid tissue, and can also be expressed on monocytes and dendritic cells; however, unlike B7RP-1, it is not found on B cells (70). Despite its homology to other B7 family members, B7-H3 does not bind to CD28, CTLA4, ICOS or PD-1 (69, 71), but when engaged with its appropriate receptor it can induce $CD4^+$ and $CD8^+$ T cells to proliferate and produce IFN-γ (72).

In addition to the growing CD28 family, there are several other costimulatory molecules that have been identified in recent years that have been shown to influence T cell responses. One of the more prominent molecular groups of costimulatory molecules is the tumour necrosis factor receptor (TNFR) family, including molecules such as OX40:OX40L, 4-1BB:4-1BBL, and CD27:CD70 (73-77). A review of these molecules is beyond the scope of this introductory chapter.

1.3.4 REGULATORY T CELLS

While central clonal deletion in the thymus and induction of anergy in the periphery have been widely accepted as mechanisms responsible for the maintenance of immune homeostasis, the role of regulatory T cells in tolerance has recently been receiving considerable attention. When the notion of a subpopulation of T cells responsible for suppression of immune responses was initially raised, it was met with substantial skepticism (78). However, over the years a considerable amount of evidence has emerged demonstrating the role of regulatory T cells (T_{REGS}) in preventing the onset of autoimmune disease, and in the maintenance of transplantation tolerance. Today, it is widely accepted that the maintenance of peripheral tolerance in several models of both autoimmunity and alloimmunity is dependent on a delicate balance between effector T cells and a unique lineage of T cells that act as "professional regulatory cells".

The fact that T_{REGS} have limited proliferative capacity in vitro has made it somewhat difficult to characterize them in detail. Nevertheless, several subsets of T_{REGS} have been identified in a variety of experimental models using different assays, and have been shown to share similar characteristics. The relationship between these subpopulations is not well understood, and whether they represent discrete subsets or diverse cell types remains unclear. Studies aimed at characterizing T_{REGS} and their mechanisms have yielded conflicting results, suggesting that T_{REGS} may exert their regulatory effects through multiple different mechanisms depending on the experimental model and the stage of differentiation.

The $CD4^{+}CD45RB^{low}$ T cell subset consisting of Tr1 and Th3 regulatory T cells can be distinguished from other $CD4^{+}$ T_{REGS} by their production of interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) to mediate their regulatory activities (79). The importance of these cytokines in maintaining immune homeostasis has been demonstrated by the development of severe autoimmune disease in IL-10 knockout mice (80) and in mice expressing a T cell-specific dominant-negative form of the TGF-β receptor II subunit (81).

With respect to the various other subsets of T_{REGS}, a common feature many of them share is the constitutive expression of the IL-2 receptor α chain (CD25), a property that is also shared with the best characterized regulatory cells in transplantation. CD4$^+$CD25$^+$ regulatory T cells occur naturally and account for approximately 5-10% of peripheral CD4$^+$ T cells in normal naïve mice (82). Sakaguchi demonstrated that the eradication of these cells results in the spontaneous development of various autoimmune diseases such as thyroiditis or gastritis in hosts that have a genetic predisposition (83). Moreover, adoptive transfer of CD4$^+$CD25$^+$ T cells has been shown to suppress the onset of autoimmune disease by autoreactive T cells in thymectomized mice (84), as well as prevent the induction of autoimmune diabetes in NOD mice (85). Like Tr1 and Th3 regulatory cells, CD4$^+$CD25$^+$ cells also produce IL-10 and TGF-β more actively than other CD4$^+$ cells; however, their regulatory activity is independent of these cytokines (79). How exactly these specialized T cells prevent rejection, as well as the signals that are specifically required for their maintenance and function, remain a matter of speculation.

One of landmark studies to report the importance of T_{REGS} in transplantation tolerance came from Qin and colleges, who demonstrated that CD4$^+$ T cells were capable of actively suppressing allograft rejection (86). Using a short course of non-depleting anti-mouse CD4 and CD8 antibody, they achieved tolerance to skin allografts in mice across minor antigens. In searching to explain how tolerance was maintained in these animals, adoptive transfer experiments were conducted using purified CD4$^+$ cells isolated from tolerant mice. Surprisingly, these cells were shown to suppress graft rejection upon adoptive transfer into naïve mice.

Since this study, the role of regulatory T cells in maintaining tolerance has been demonstrated in several rodent models (87-89), including those in which tolerance is induced through the blockade of costimulation (90, 91). Moreover, certain important principles regarding the role of T_{REGS} in transplantation tolerance have emerged. First is the concept of **linked suppression** of graft rejection (92-95). This refers to the phenomenon where tolerant mice, although capable of rejecting third party allografts, can fully accept a graft from an F1 cross between the donor and a third party. Acceptance of this F1 graft can then lead to full tolerance of the third party graft, without any further immunomodulation of the recipient. This indicates that donor-specific regulatory T cells can be directed to induce tolerance to third party specific T cells provided that both donor and third party antigens are "linked"; that is, brought closely together, either on the same APC or target tissue. The concept of linked suppression may have important clinical implications since it may not be necessary to induce tolerance to every transplantation antigen in order to achieve graft acceptance (96). This may be of particular relevance in clinical islet transplantation where diabetic recipients presently require two, or sometimes even three, donor pancreata to achieve insulin independence (97). A second important principle that has emerged is that tolerance mediated by T_{REGS} is infectious (87, 98, 99). The term **"infectious tolerance"** refers to the phenomenon whereby the adoptive transfer of T_{REGS} into naïve animals not only suppresses naïve T cells, but, in the presence of an allograft, can induce naïve $CD4^+$ T cells to differentiate into T_{REGS}. Interestingly, these second-generation T_{REGS} have the ability to suppress rejection by further cohorts of naïve T cells. Once initiated, infectious tolerance can take place over several generations of naïve recipients, generating new cohorts of T_{REGS} in each subsequent generation. This phenomenon provides an explanation for the persistence of allograft tolerance despite the continuous supply of new alloreactive T cells from the host thymus (88). A third principle of importance is that T_{REGS} are not simply a by-product of allograft tolerance, but are necessary for the maintenance of the tolerant state. This has been confirmed by the fact that elimination of

CD4$^+$ T cells in tolerant animals has resulted in graft rejection, mediated by CD8$^+$ T cells that have been freed from the suppressive effects of regulatory T cells (100).

The field of regulatory T cells in transplantation tolerance is expanding at an incredible pace and offers the potential for significant therapeutic advantages as new insights into mechanisms are uncovered. Further understanding of the function of T_{REGS} may lead to a means of inducing or expanding regulatory T cells in vivo and in vitro. This concept is currently being explored by the Immune Tolerance Network and could have tremendous implications in the field of transplantation tolerance.

1.4 MECHANISMS FOR ACHIEVING TRANSPLANTATION TOLERANCE

The mechanisms that form the basis of acquired transplantation tolerance may be described in terms of the same mechanisms that allow the immune system to maintain a state of immune hemostasis. These mechanisms can be divided into two categories, namely **central and peripheral** mechanisms of tolerance. Central tolerance is a well-described mechanism that involves the clonal deletion of self-reactive T cells in the thymus. Immature T cells from the bone marrow travel to the thymus where they encounter self peptides coupled to MHC molecules (101). The fate of these immature T cells is determined by their affinity for the peptide-MHC complexes they encounter. If the TCR forms a weak interaction with the complex, it does not receive signals to prevent spontaneous apoptosis resulting in the elimination of the T cell. Strong interactions between the T cell and the peptide-MHC complex are also lethal and lead to death of T cells in a process known as negative selection. Conversely, interactions of intermediate affinity are stimulatory and result in the maturation of these immature T cells for the immune repertoire in a process called positive selection (102). Since not all self-antigens are present in the thymus for efficient negative selection, a small number of self-reactive T cells escape into the periphery. Therefore, the "leakiness" of central tolerance necessitates the existence of peripheral mechanisms that regulate the fugative self-reactive T cells to maintain immune hemostasis (103, 104).

1.4.1 PERIPHERAL TOLERANCE

Peripheral tolerance is mediated by a number of separate mechanisms that act as safeguards against self-reactive T cells (Figure 1-9). These include immunologic ignorance, anergy (non-responsiveness), deletion, inhibition and active suppression (102, 105, 106).

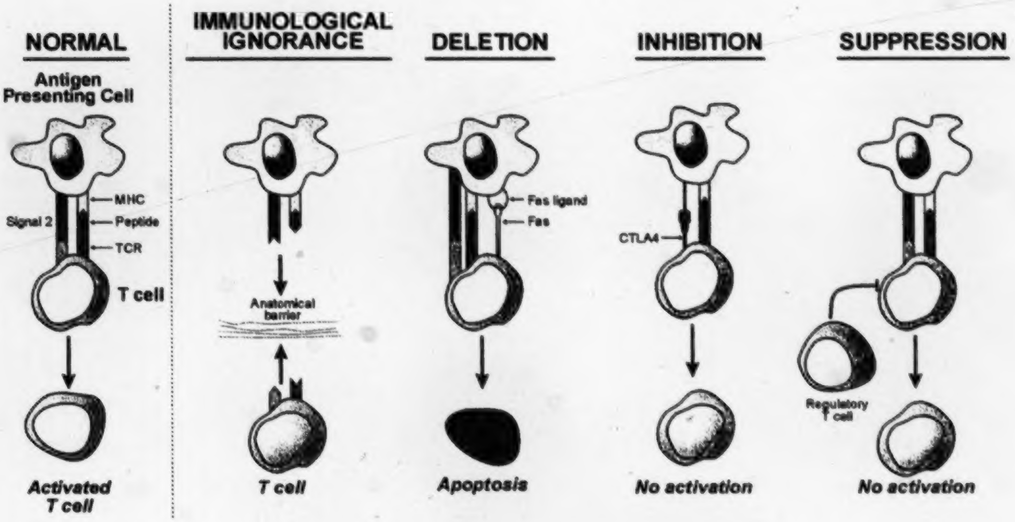
NORMAL
IMMUNOLOGICAL IGNORANCE
DELETION
INHIBITION
SUPPRESSION
Antigen Presenting Cell
MHC
Signal 2
Peptide
TCR
T cell
Anatomical barrier
Fas ligand
Fas
CTLA4
Regulatory T cell
Activated T cell
T cell
Apoptosis
No activation
No activation

Figure 1-9: Peripheral mechanisms of tolerance.

T cells are kept in ignorance when the level of antigen is below the threshold to stimulate an immune response, or when self-antigen is kept behind cellular or vascular barriers (e.g., the blood-brain barrier). Anergy refers to a state of unstable metabolic arrest that usually results in apoptosis. This phenomenon is induced when a T cell receives an antigenic signal without the necessary costimulatory second signal (32-34). Deletion occurs either passively when activated T cells are deprived of growth factors, or actively through activation-induced cell death (AICD) by autoligation of the FAS-FAS ligand pathway on T cells (107). Inhibition arises when primed T cells are switched off through the activation of coinhibitory signals (e.g., CTLA4 on T cells binding to B7-1 or B7-2 on APCs) (108). Finally, active suppression occurs when regulatory T cells inhibit the induction of effector functions either by producing inhibitory cytokines or by interfering with receptor signaling pathways (82).

Peripheral mechanisms of tolerance are not mutually exclusive and their relative involvement in the maintenance of self-tolerance is not entirely well understood (109). Nevertheless, numerous promising tolerance-inducing strategies involve the manipulation of these peripheral mechanisms in an attempt to establish a state of graft acceptance. One of the most intensely studied approaches is the deletion of the donor specific T cells at the time of transplantation. The high frequency of alloreactive T cells in the recipient's peripheral lymphoid compartment poses a formidable barrier to achieving long-term graft acceptance. The blockade of surface molecules that deliver critical signals to activate the T cell has emerged as a powerful strategy to specifically target the alloreactive population at the time of transplantation. This approach has evolved from initial studies using antibodies specific for the T cell co-receptor CD4 (110-112) to the more sophisticated monoclonal antibodies that block the delivery of costimulation. An important consideration in deletional strategies is the need to contend with the continual emigration of new donor-specific T cells from the recipient thymus. Therefore, although reducing the frequency of alloreactive T cells is critically important for graft acceptance, the simultaneous amplification of regulatory mechanisms is equally important for the maintenance of tolerance (113). By reducing the frequency of donor-specific T cells, regulatory mechanisms can then exert a functional dominance over the remaining peripheral allospecific T cells and the newly generated allospecific T cells from the thymus (114, 115). In this way, the delicate balance between rejection and acceptance remains tipped towards the maintenance of the tolerant state (Figure 10).

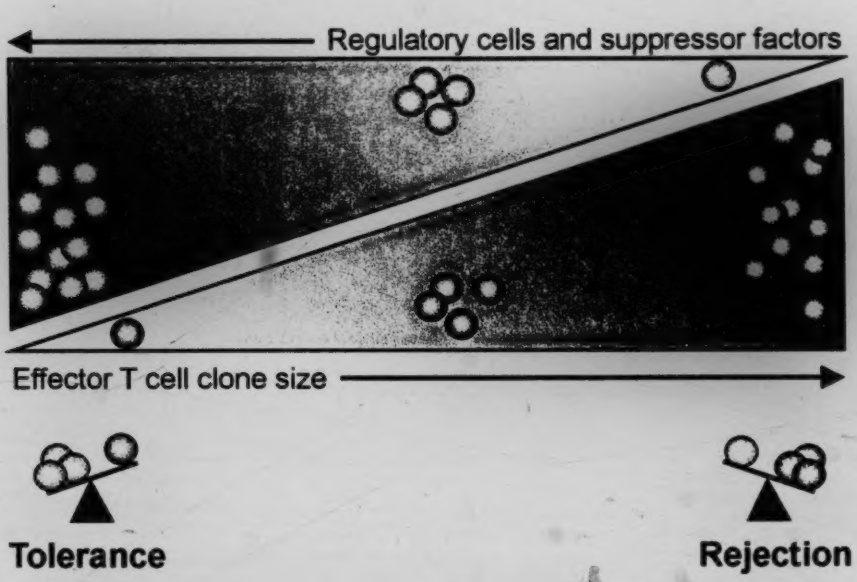
Regulatory cells and suppressor factors
Effector T cell clone size
Tolerance
Rejection

Figure 1-10: The delicate balance between pathogenic effector cells and tolerogenic regulatory cells
In the presence of large numbers of effector cells, regulatory mechanisms cannot suppress the effector response, leading to rejection of the allograft. Conversely, in the presence of large numbers of regulatory cells, effector mechanisms cannot reject the allograft, resulting in the maintenance of tolerance.

1.4.2 CENTRAL TOLERANCE - ESTABLISHMENT OF MIXED CHIMERISM

Chimerism, in the context of transplantation, refers to the existence in a transplant recipient of hematopoietic elements from a donor that is allogeneic to the recipient (116). When tolerance was experimentally induced for the first time by

Medawar's group, it was accomplished by the inoculation of neonatal mice with a suspension of allogeneic hematopoietic cells (9). These studies demonstrated that the establishment of hematopoietic chimerism can induce transplantation tolerance, such that when an allograft from the same donor strain as the innoculum is transplanted into the chimeric recipient, it is regarded as "self" and is permanently accepted.

The establishment of chimerism in the setting of an established immune system requires ablation of the preexisting immune system with subsequent re-constitution with donor hematopoietic cells in the form of a bone marrow transplant (BMT) (117). This strategy leads to a state marochimerism, which can be characterized as either **"full" chimerism** or **"mixed" chimerism**. Full hematipoietic chimeras are defined by a state in which all hematopoietic cells in the recipient are of donor origin. This is achieved through a combination of complete ablation of the lymphohematopoietic system of the recipient followed by transplantation of donor-alone hematopoietic cells. The result is complete or near-complete reconstitution of the recipient's bone marrow compartment with donor hematopoietic cells (118). Alternatively, mixed chimerism refers to the co-existence of both donor and host hematopoietic cells in the recipient. Establishing a mixed chimera does not require complete myeloablation of the recipient's bone marrow and, therefore, can be achieved with less lethal methods of "conditioning" prior to BMT (118).

With respect to tolerance induction, the establishment of mixed chimerism has significant advantages over full chimerism. One of the most important advantages is that mixed chimerism can be accomplished through **nonmyeloablative strategies**, thereby minimizing the morbidity associated with host myeloablaive conditioning (117). Additionally, mixed chimeras are theoretically more immunocompetent than full chimeras across MHC barriers (119). The immunological basis for this difference is explained by the disparity between the MHC specificity of the T cells exiting the thymus, which is of recipient origin, and the MHC expressed by the antigen-presenting cells in the periphery, which are entirely of donor origin in full chimeras. Therefore, there exists a discrepancy between the MHC expressed on the APC and the MHC for which the T-cell was selected, resulting in the inability to generate an appropriate immune response. However, due to some sharing of specificities, relatively weak immune responses can be generated in full chimeras. Conversely, in mixed chimeras, immune responses are not impeded since there is a continuous supply of host APC's that can interact with host-restricted T-cells (117, 120).

Another advantage of mixed chimerism over full chimerism arises from the fact that intrathymic clonal deletion of host-reactive T-cells is significantly more effective in mixed chimeras. Although clonal deletion of self-reactive T-cells can be achieved through nonhematopoietic thymic stromal cells, it is far more effective in the presence of hematopoeitic cells, especially dendritic cells (121, 122). Therefore, due to the lack of host-derived hematopoeitic cells seeding the thymus in full chimeras, clonal deletion of host-reactive T-cells is dependent upon thymic stromal cells alone, which is substantially less effective. It is important to note that while these disadvantages have been well described, the actual magnitude of these effects in the host remains controversial. At present, these limitations represent a theoretical concern only, and do not appear to manifest to any appreciable extent clinically in patients undergoing BMT for hemoglobinopathies.

In addition to the above two forms of macrochimerism, a third type of chimerism exists and is known as **microchimerism**. According to Starzl, microchimerism is a naturally occurring phenomenon after organ transplantation, and is the result of persisting donor hematopoeitic cells originally present in the transplanted organ (123). In a microchimera, donor hematopoietic cells exist in minute quantities, which can only be detected by highly sensitive techniques such as polymerase chain reaction (PCR) (118). With respect to transplantation tolerance, it has been suggested that microchimerism can lead to tolerance in human and animal models (123). Earlier studies supported this hypothesis by demonstrating that rejection could be increased by depletion of hematopoietic cells from the donor organ prior to transplantation (124, 125). However, other studies suggested that microchimerism does not play a causal role in the maintenance of tolerance based on findings that a) recipients undergo rejection despite persistence of donor-specific microchimerism (126-128), and b) recipients can accept cardiac allografts long-term despite depletion of donor hematopoietic cells (129). More recently however, it has been suggested that microchimerism is a "double-edged sword" that can result in both immunity and tolerance depending on the maturity of the recipient immune system. In immunologically mature recipients, microchimerism results in allograft rejection, whereas in immature hosts it leads to a state of tolerance that is specific to antigens expressed by the donor chimeric cells only (130).

1.4.3 MIXED CHIMERISM IN TRANSPLANTATION TOLERANCE

The development of mixed chimerism in an animal model begins with the deletion of the existing mature donor-reactive T cell population and the creation of sufficient hematopoeitic space for the new marrow to engraft. The former can be accomplished with cytotoxic antibodies directed against specific T-cell receptors or with costimulatory blocking agents, while the latter is achieved by total body irradiation (TBI) or cytotoxic drugs. Although very effective, these strategies are associated with significant morbidity owing to the complete or near-complete destruction of all immunocompetent T cells, as well as to the toxicities associated with the agents themselves.

Once the peripheral immune system has been cleansed of its T cell population and sufficient hematopoeitic space has been created, the recipient's bone marrow compartment is reconstituted through intravenous infusion of self and allogeneic bone marrow. The donor and recipient stem cells co-exist and give rise to hematopoietic cells of both allogeneic and recipient lineages. Once released into the peripheral circulation, these cells are distributed to various hematopoietic compartments of the recipient including the thymus, the site where tolerance is maintained through the naturally occurring process of central clonal deletion (102). Therefore, in the case of mixed chimeras, hematopoietic cells from both the recipient and donor seed the thymus and hence mediate the elimination of both host-reactive and donor-reactive T-cells by negative selection (121, 131, 132). The result is a newly constituted T cell repertoire that is tolerant towards both the donor and the host (Figure 1-11). Khan et al. have eloquently demonstrated that intrathymic clonal deletion, and not peripheral suppression or anergy, is the primary mechanism for the maintenance of tolerance in mixed allogeneic chimeras (133). As long as the donor hematopoietic stem cells remain engrafted in the recipient's bone marrow, there will be a continuous supply of donor antigen presenting cells to the thymus, and hence the maintenance of this tolerant state indefinitely (118).

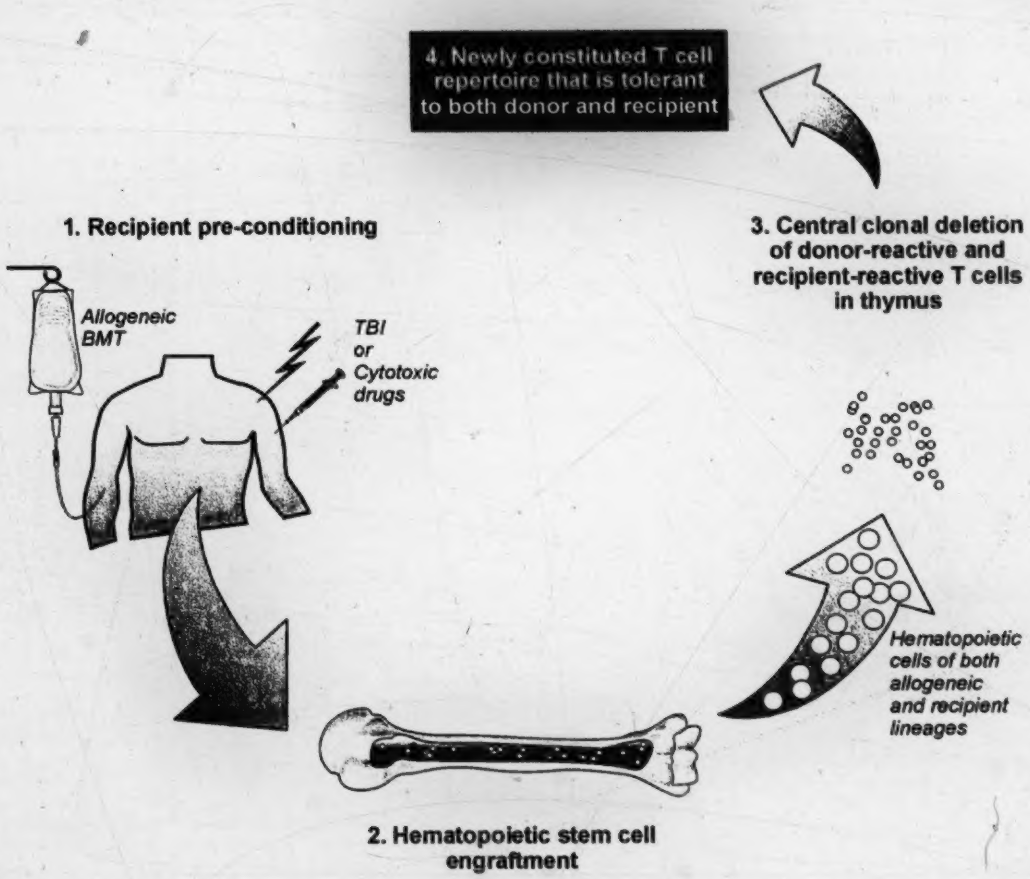
4. Newly constituted T cell repertoire that is tolerant to both donor and recipient
1. Recipient pre-conditioning
Allogeneic BMT
TBI or Cytotoxic drugs
3. Central clonal deletion of donor-reactive and recipient-reactive T cells in thymus
Hematopoietic cells of both allogeneic and recipient lineages
2. Hematopoietic stem cell engraftment

Figure 1-11: Principles underlying the establishment of stable mixed chimerism and transplantation tolerance.

Tolerance induction through the development of mixed chimerism is widely acknowledged as a reliable and robust method of tolerance induction. A testament to the strength of tolerance in mixed chimeras is that these models demonstrate endurance to the most rigorous tests for transplantation tolerance, such as permanent primary skin allograft acceptance. Moreover, as opposed to peripheral mechanisms for inducing tolerance, such as anergy and suppression of alloreactive T cells, tolerance through mixed chimerism is based on the central mechanism of intrathymic clonal deletion of donor-reactive T cells. In peripheral strategies, donor-reactive T cells are not eliminated but prevented from effectively engaging their targets. Therefore, since donor-reactive cells continue to persist in peripheral strategies, tolerance may be overcome under certain conditions, rendering this form of tolerance less sturdy and less reliable (131, 134). An important caveat to the strength of tolerance through the induction of mixed chimerism is that it is critically dependent upon the maintenance of the chimeric state. A loss of chimerism in the host almost certainly results in the subsequent rejection of the donor graft, emphasizing the importance of long-term donor bone marrow engraftment.

The application of bone marrow transplantation for tolerance induction in organ transplant recipients has tremendous potential to be translated to the clinic. In fact, the effectiveness of this approach in patients has already been established, in light of successful cases of bone marrow transplant recipients with established donor chimerism who have been able to accept a renal transplant from the same donor without any immunosuppression (135-138). However, despite these reports, the establishment of mixed chimerism in human recipients for organ transplantation is not a practically viable option for most patients at this time. The major limitations lie in the severe toxicity of the myelosuppressive host conditioning required for allogeneic bone marrow engraftment, and the fact that exhaustive depletion of T cells of the host leads to a period of severe immunoincompetence and vulnerability to life-threatening ailments. Furthermore, the risk of engraftment failure and the risk of graft-versus-host disease (GVHD), even when partial HLA barriers are transgressed, are other significant obstacles to the clinical application of mixed chimerism strategies (117, 118). Although the risk of GVHD can be greatly reduced by eliminating the mature T cells from the allogeneic bone marrow, the more devoid the donor bone marrow is of T cells, the higher the risk for engraftment failure, which can be fatal in recipients of supralethal doses of TBI and/or chemotherapy (116). Moreover, the clinical application of mixed chimerism also has considerable limitations with respect to the inflexible logistics of human organ transplantation. Since minimizing the delay in transplantation of harvested organs into recipients is of paramount importance, conditioning of patients with bone marrow transplantation would need to be completed in a very narrow window of time. In this regard, islet transplantation could serve as a primary test bed for novel chimerism protocols since optimization of the recipient could be accomplished while islets are kept in culture. Nevertheless, significant challenges remain and considerable research is still required in the development of less toxic, one-day BMT protocols before chimerism strategies can be safely and effectively applied in transplant patients.

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CHAPTER TWO

ADVANCES IN PANCREATIC ISLET TRANSPLANTATION IN HUMANS

Sulaiman A. Nanji[1] and A.M. James Shapiro[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada

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2.1 INTRODUCTION – THE DIABETES BURDEN AND RATIONALE FOR ISLET TRANSPLANTATION

Diabetes affects more than 200 million people worldwide, representing the third most common disease and forth leading cause of death in North America (1). The incidence of diabetes is increasing rapidly, with 30,000 new type 1 diabetics annually in North America (2, 3). Diabetes (mainly type 2) poses a colossal financial burden to our global society, comprising nine to 15% of healthcare expenses in developed countries. The mainstay treatment for type 1 diabetic patients is chronic insulin injection. While exogenous insulin therapy has dramatically reduced mortality from diabetes, patients often succumb to the long-term sequelae of diabetic angiopathy, either in the form of nephropathy, neuropathy or retinopathy. Maintaining rigorous glycemic control with intensive insulin therapy has been shown to delay and sometimes prevent the progression of these complications, but patients are at risk of severe and sometimes fatal hypoglycemic events (4, 5). Although insulin pumps and implantable insulin-secreting devices are a promising approach to improved glucose homeostasis, the development of reliable and accurate glucose sensor technology has been a limiting factor. A more physiologic approach to correct the diabetic state is the transplantation of insulin-producing tissue.

At present, vascularized pancreas transplantation reliably restores normoglycemia and maintains long-term glucose homeostasis. It has been shown to improve quality of life (6, 7) and even reverse some secondary complications of diabetes (8). Simultaneous pancreas and kidney transplantation is presently considered the standard of care for selected patients with type 1 diabetes with end-stage renal failure (9). Although pancreas transplantation achieves insulin-independence in greater than 80% of patients beyond 1 year (10), it remains a significant surgical procedure with substantial morbidity and occasional mortality (11). Numerous studies have reported on the beneficial effects on survival, quality of life and impact in stabilization and even reversal of secondary diabetic complications when a pancreas is transplanted into a patient with end stage renal failure in addition to a kidney (12-14). A recent controversial report by Venstrom and colleagues has raised the possibility that patient survival could be compromised after pancreas alone or pancreas after kidney transplants, compared to patients awaiting this procedure in the USA (15). This study has recently been brought in to question, as it excluded patients with modestly impaired renal function – the group that might be expected to have an increased risk of mortality on the waiting list.

In view of the risks associated with surgery and long-term immunosuppressive drug therapy, pancreas transplantation is largely reserved for diabetic patients with clinically significant diabetic complications, where the severity of their disease justifies accepting the risks of the procedure and immunosuppression. Therefore, with the exception of rare patients with severe, labile forms of diabetes, pancreas transplantation is not a practical option for young diabetic patients who have not yet developed diabetic complications.

A promising alternative is the transplantation of islet cells isolated from donor pancreata and embolized into the recipient liver via the portal vein (Figure 2-1). Compared to pancreas transplantation, islet transplantation is technically much simpler, has low morbidity, and offers the opportunity for storage of the islet graft in tissue culture or cryopreservation for banking. Moreover, the fact that islets can be kept in culture provides a unique opportunity to immunologically manipulate the islet graft, as well as optimize recipient conditioning prior to transplantation, thereby facilitating tolerance induction. The low morbidity of the procedure and the potential for tolerance induction make islet transplantation a promising strategy for correcting diabetes in young patients, including children (16), prior to the establishment of secondary complications.

While enthusiasm for clinical islet transplantation began in the early 1970s, its application was significantly limited, largely due to poor quality, low-yield islet preparations and ineffective immunosuppression. Recently, however, clinical outcomes in islet transplantation have improved dramatically, making it an effective therapy for selected patients with type 1 diabetes.

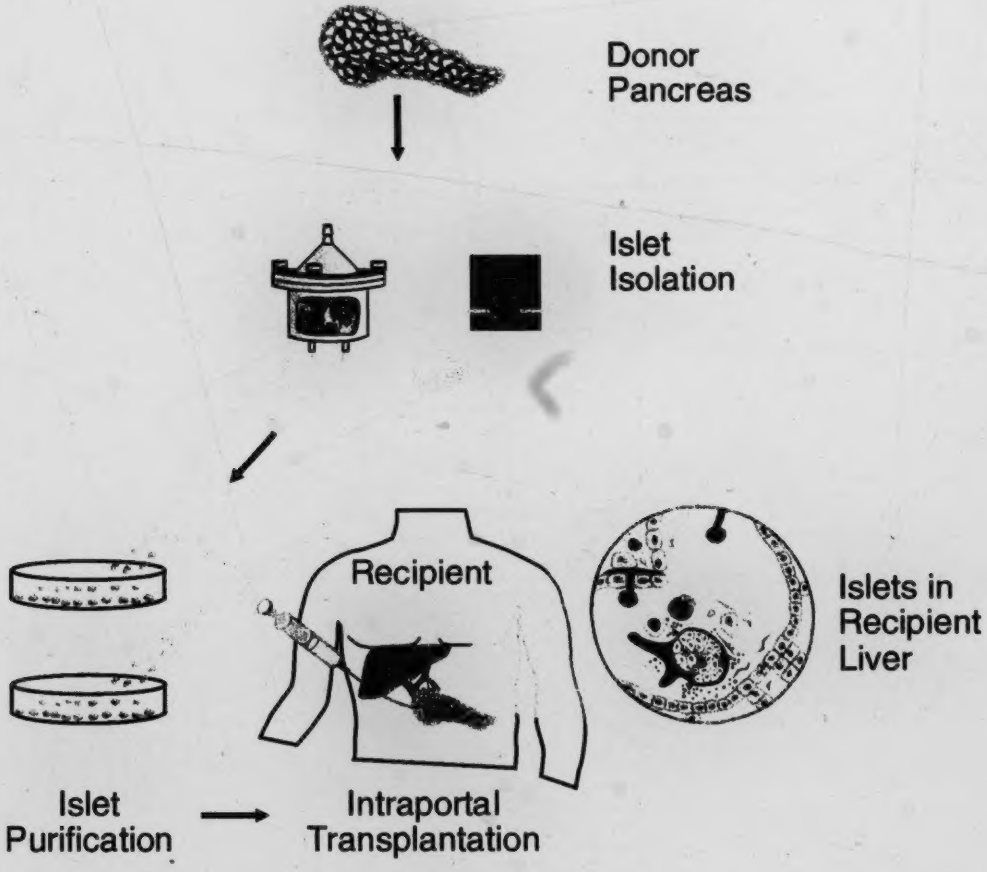

Donor
Pancreas
Islet
Isolation
Recipient
Islets in
Recipient
Liver
Islet
Purification
Intraportal
Transplantation

Figure 2-1: Steps involved in islet transplantation.

2.2 ISLET TRANSPLANTATION: EARLY EFFORTS TO PRESENT SUCCESS

The concept of transplanting pancreatic tissue for the treatment of diabetes dates back more than a century ago, well before the discovery of the immune system or the development of anti-rejection therapy. The connection between the pancreas and diabetes was first described in 1889 by von Mering and Minkowski who observed that pancreatectomized dogs developed hyperglycemia and glycosuria (17). Five years later, Dr. Watson-Williams at the Bristol Infirmary in England performed the first clinical pancreatic tissue transplant by implanting three pieces of freshly slaughtered sheep's pancreas into the subcutaneous tissues of a young boy dying from diabetic ketoacidosis (18). Without immunosuppression the xenograft was fated to fail, however, it did lead to an improvement in the boy's glucose excretion prior to his death three days after the transplant. In 1916, Pybus of Newcastle-on-Tyne reported a mild reduction in glucose excretion in one of two diabetic patients transplanted with fragments of human cadaveric pancreatic tissue (19). Four years later at the University of Toronto, Frederick Banting discovered that ligation of the pancreatic duct in dogs led to enhanced recovery of the "internal secretions" of the pancreas (20). Subsequent studies by Banting, Best, Collip and MacLeod led to the discovery of insulin (21, 22), and its rapid introduction into clinical practice revolutionized the treatment of diabetes.

While mortality from diabetes was radically reduced with exogenous insulin therapy, the development of secondary complications became strikingly apparent as patients lived longer with their disease (23-25). Research in pancreatic tissue transplantation was revived when it was evident that insulin could not prevent these potentially fatal complications. In 1966, Kelly and Lillehei at the University of Minnesota performed the first vascularized pancreas transplant (26). Initial series were associated with dismal morbidity and mortality (27) and the concept of transplanting just the islets instead of the whole pancreas was viewed as an attractive alternative.

Progress in rodent models in the early 1970s with improvements in the islet isolation procedure (28), and subsequent reports of successful reversal of chemical diabetes in rodents receiving islet isografts (29-31), generated excitement in the clinical application of this approach. However, early attempts at replicating rodent studies in large animal models were disappointing, largely due to the inability to isolate sufficient quantities of optimal islets for transplantation. As a result, researchers attempted to transplant pancreatic fragments instead of isolated islets in order to deliver a sufficient islet mass to achieve insulin independence. Based on initial success in dogs (32, 33) clinical trials were attempted, culminating in the first series of clinical islet allotransplants by Najarian and colleagues in 1977 at the University of Minnesota (34). Initial clinical studies were disappointing as implantation of pancreatic tissue fragments into the peritoneal cavity or embolized to the liver was essentially ineffective. None of the patients were rendered insulin independent and only some had reduced insulin requirements for limited periods (34). Moreover, although the liver appeared to be the optimal site for islet transplantation, the injection of larger volumes of tissue was associated with significant complications including portal vein thrombosis, portal hypertension and even mortality (35, 36). It was clear that more purified islet preparations would be required in order to improve safety for islet transplantation to become a clinical reality.

Several advances such as the Ricordi digestion chamber (37), the COBE continuous purification system (38), controlled pancreatic distension with the digestive enzyme collagenase (39), and purified enzyme blends with low endotoxin levels (40), all contributed to improvements in obtaining higher-yield, better quality islet preparations. Nevertheless, clinical outcomes remained disappointingly poor. Between 1974 and

1999, over 450 cases of islet allotransplantation for the treatment of type 1 diabetes were reported to the Islet Transplant Registry, with less than 10% of patients achieving insulin independence for longer than one year; although 28% had sustained C-peptide secretion (41-43). The lack of clinical success was attributed to several factors including: inadequate islet transplant mass; ineffective prophylaxis against allograft rejection and autoimmune recurrence; and continued use of toxic, diabetogenic immunosuppressive agents such as cyclosporine and glucocorticoids (44-46). In consideration of these limitations, a new protocol was implemented in Edmonton, Canada in 1999 that radically changed the face clinical islet transplantation. The initial series of seven type 1 diabetic patients all achieved and maintained insulin independence beyond one year, demonstrating for the first time that islet transplantation could be as effective at achieving insulin independence as whole pancreas transplantation (47). The success of the "Edmonton Protocol" has been attributed to two key modifications from previous clinical trials. First, patients received an adequate number of high-grade islets prepared from an average of two donor organs. Second, more potent but less diabetogenic, steroid-free anti-rejection therapy was achieved using a novel combination of sirolimus, low-dose tacrolimus and an anti-interleukin-2 receptor monoclonal antibody (anti-IL-2R mAb).

Since the release of the early Edmonton results, considerably more experience has been accrued both in Edmonton and at other centers worldwide. At the University of Alberta, a total of 66 patients have now received islet-alone transplants. Most patients continue to require two islet infusions in order to provide adequate engraft mass (approximately 12,000 IE/kg islet mass, based on the recipient body weight). Of patients undergoing completed islet transplants, 82% remain insulin free by the end of one year (Figure 2-2). There is some fall off in insulin independence, with 70% remaining insulin free at two years and 50% free at three years post transplant. Most patients that return to insulin continue to secrete endogenous insulin (and C-peptide) in sufficient amounts to continue to stabilize risk of hypoglycemic reactions or of glycemic lability, and 88% of patients continue to demonstrate islet function out to five years post transplant. Islet transplantation has proven to be remarkably successful in stabilizing glucose control to a degree that is vastly superior to even intensive insulin therapy, and patients typically demonstrate normalization of HbA1C (48).

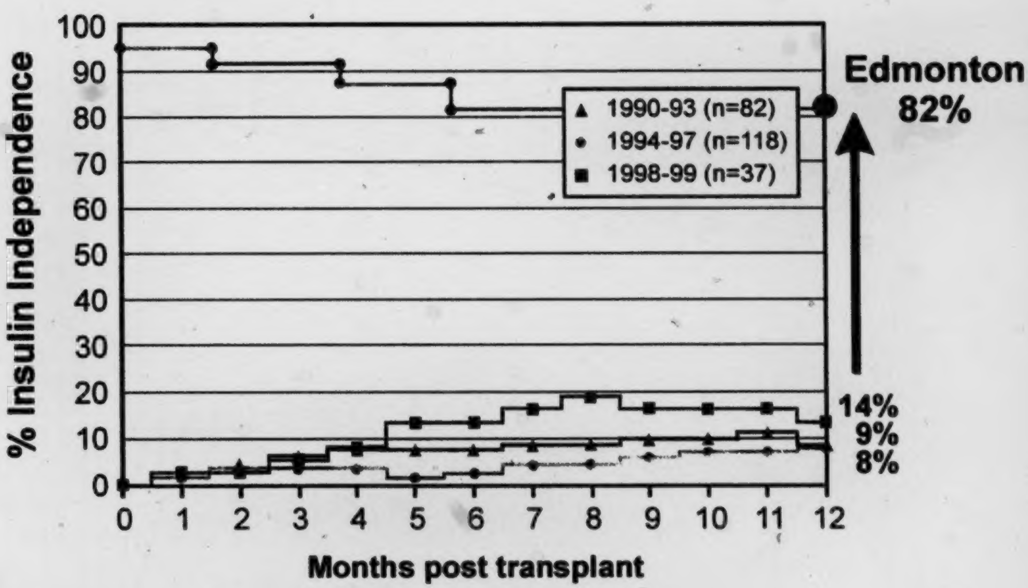
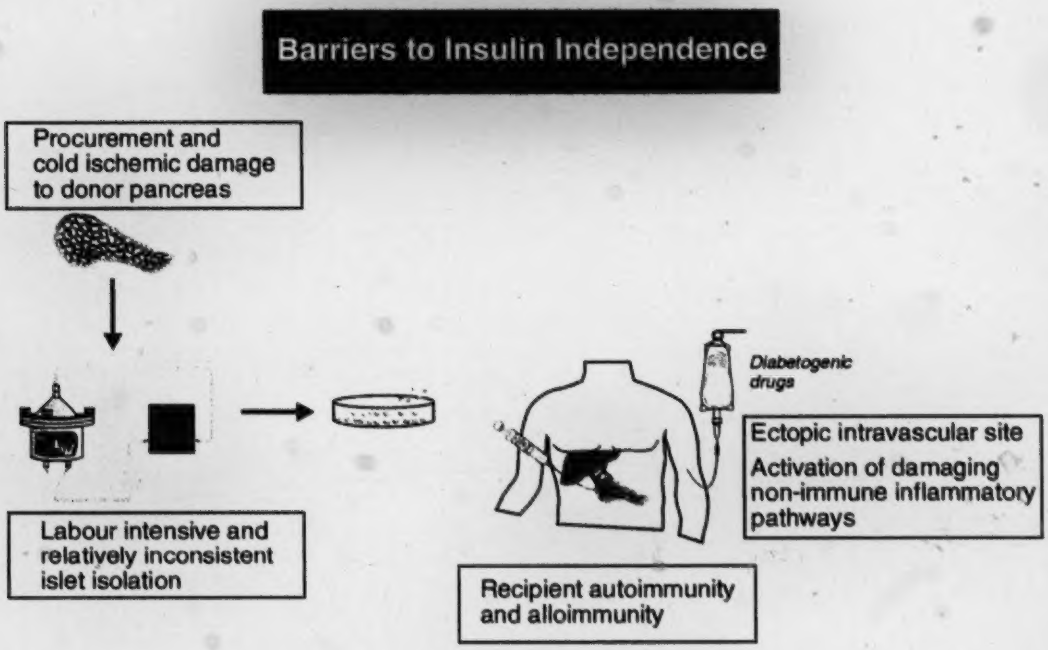


Figure 2-2: Improvements in outcomes in clinical islet transplantation. Dramatically higher rates of insulin independence were achieved in patients treated with the Edmonton Protocol, compared to previous reports in the international Islet Transplant Registry (Adapted form A.M.J. Shapiro, PhD Thesis 2001, with kind permission).

An international multicenter trial of the Edmonton Protocol was recently completed by the Immune Tolerance Network in nine sites, and demonstrated that the original Edmonton findings could be replicated, at times to a very high level of success, depending on the experience of the site (49). Worldwide there have now been over 350 patients treated since 1999, and increasing momentum and focus on the remaining challenges of islet isolation, alternative insulin-secreting regulated sources, better immunosuppression with less side effects and the possibility of immunological tolerance continue to drive the field forward.

2.3 RECENT ADVANCES IN ISLET TRANSPLANTATION

Over the past few years there has been tremendous progress in clinical islet transplantation, from refinements of the Edmonton Protocol to novel strategies for improved islet isolation, implantation and recipient immunosuppression. The following sections address some of the most significant barriers in clinical islet transplantation (Figure 2-3) and highlight the most recent developments towards achieving higher rates of insulin independence.



Barriers to Insulin Independence
Procurement and cold ischemic damage to donor pancreas
Labour intensive and relatively inconsistent islet isolation
Diabetogenic drugs
Ectopic intravascular site
Activation of damaging non-immune inflammatory pathways
Recipient autoimmunity and alloimmunity

Figure 2-3: Barriers to achieving insulin independence in islet transplantation.

2.3.1 PANCREAS PROCUREMENT AND ISLET ISOLATION

One of the most critical areas of ongoing research is the islet isolation procedure, which remains highly labour intensive, expensive and relatively inconsistent. Even the highest-grade preparations only recover about 20 to 50% of the potential islet mass (50). Moreover, rates for successful islet isolation at leading centers vary from 25 to 75%, depending largely on the quality of the pancreas, the amount of cold storage, and the heterogeneity of collagenase preparations. To address these concerns, several strategies have evolved to enhance islet yields and ensure reproducibility of the procedure.

The quality of the donor pancreas depends largely on donor factors such as age, body mass index, serum glucose levels and hemodynamic stability (51). However, principles in pancreas procurement such as atraumatic manipulation of the pancreas, immediate in situ cooling of the pancreas, and rapid transport of the organ to the islet isolation laboratory have been shown to minimize both warm and cold ischemic injury, stabilize endogenous enzyme activity, and lead to significantly improved islet yields and viability (52). A further concern that has a significant impact on islet isolation yield is the duration of cold ischemia (53-55), given that the donor pancreas typically requires to be transported over long distances to centralized islet isolation centers. There have been no reports of successful single-donor islet transplants with cold storage times in excess of 10 hours (43), and others have demonstrated that longer cold ischemic times reduce post-transplant islet function (56). One of the most remarkable advances to overcome this concern has been the introduction of a "two-layer" cold storage method using perfluorocarbons (PFCs) and standard University of Wisconsin preservation solution (56-58). PFCs have an extremely high affinity for oxygen, which diffuses into the preserved pancreas, thereby maintaining membrane integrity and reducing ischemic cell swelling

(59, 60). The two-layer method has been shown to reverse the damaging effects of warm ischemia, increase islet yields and improve islet engraftment (61-64). This method also has the potential to expand the donor pool by salvaging pancreata that would otherwise be unusable (65).

Although patients in the initial Edmonton series were transplanted with islets immediately after isolation, some centers are currently maintaining islets in culture prior to transplantation. Culturing islets does not appear to have detrimental effects on viability and function (66) and provides opportunities for pre-transplant conditioning of the recipient, immunological manipulation of the islet graft to promote engraftment and prevent rejection, and for identifying the best matched recipients. In addition, through the development of specific culturing conditions, the Miami group has demonstrated that islets can be shipped to remote transplant centers without compromising viability (67). Moreover, maintaining islets in culture enhances islet purity, which in turn improves engraftment and safety while reducing graft immunogenicity (68-70). However, others have argued that islets still attached to acinar tissue, so called mantle islets, are superior to completely pure islet preparations. This is based on the observation that exocrine tissue can exert trophic effects on precursor cells in the ductal epithelium of a less pure sample, thereby promoting neogenesis of beta cells (71, 72).

2.3.2 ISLET ENGRAFTMENT

The loss of viable islets is a significant concern not only during the isolation and purification process (73), but also when embolized into the portal vein of the recipient liver. Based on metabolic tests in post-transplant recipients, it is estimated that only 25 to 50% of the implanted islet mass actually engrafts in the patient (48). Recently, the Uppsala group in Sweden have shown that human islets exposed to ABO-compatible blood triggers an "instant blood mediated inflammatory reaction" (IBMIR), characterized by activation of platelets and the coagulation and complement systems, leading to islet damage by clot formation and leukocyte infiltration (74). Further investigation into the mechanisms of this phenomenon revealed that tissue factor and thrombin play critical roles in mediating IBMIR, indicating that strategies to block binding of these factors may have considerable therapeutic potential in islet transplantation (75, 76). For example, the use of low molecular weight dextran sulfate has been shown to significantly abrogate IBMIR in an in vitro tubing loop assay, as well as promote the survival of islets in recipient mice treated with this agent (77). In recent years, other experimental strategies have been developed to enhance islet engraftment. For instance, anti-inflammatory treatment with TNF-alpha-receptor antibody (78), as well as anti-oxidant therapy with nicotinamide (79, 80), vitamin D3 (81, 82), pentoxiphylline (83) or cholesterol lowering agents pravastatin or simvastatin (84, 85), have all demonstrated positive impact in the pre-clinical setting, and suggest a potential role in future clinical trials designed to improve islet engraftment. By reducing inflammatory reactions, these agents may also have the added benefit of decreasing the alloreactivity of the graft, thereby facilitating the success of tolerance promoting strategies.

2.3.3 RECIPIENT IMMUNOSUPPRESSION

Perhaps the most critical area for further investigation in islet transplantation is immunosuppression. The anti-rejection regimen in the Edmonton Protocol is arguably one of the most important recent developments in making islet transplantation a clinical reality. The tri-site combination therapy effectively provides potent immunosuppression to overcome both alloimmune rejection and autoimmune recurrence while minimizing toxicity to the islet graft, thereby avoiding the diabetogenic impact on a limited β-cell reserve (Figure 2-4). While the risk of malignancy, post-transplant lymphoma and life-threatening sepsis has been exceedingly low in patients under this immunosuppressive regimen, fears of these complications limit broader application in patients with less severe forms of diabetes including children. Furthermore, medication side effects have included severe mouth ulceration, hypertension, weight-loss, anemia, elevated cholesterol, accelerated nephropathy and diabetogenicity (48). Therefore, although outcomes in islet transplantation have significantly improved, extensive refinements in

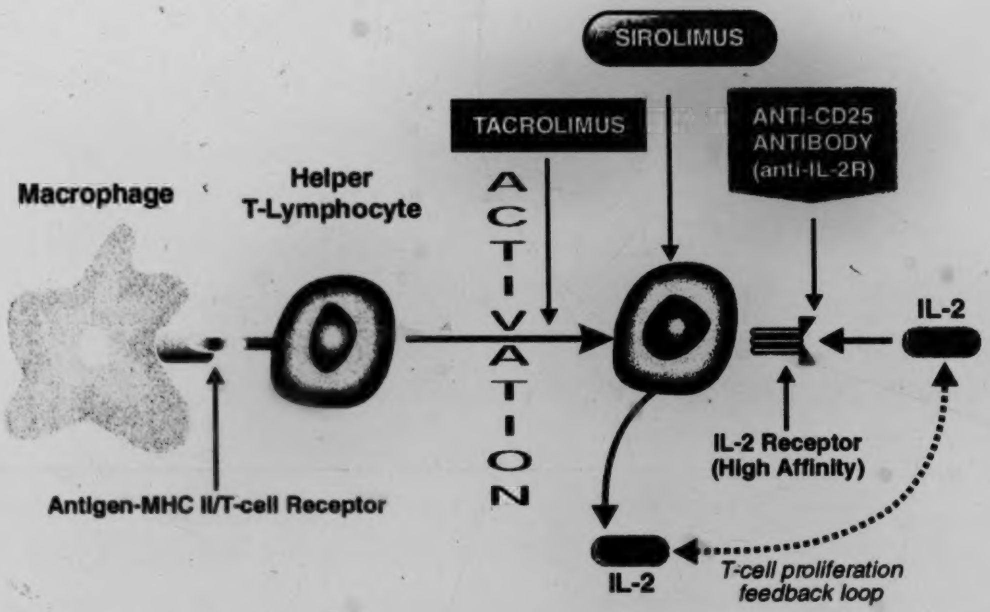

SIROLIMUS
TACROLIMUS
ANTI-CD25 ANTIBODY (anti-IL-2R)
Macrophage
Helper T-Lymphocyte
ACTIVATION
IL-2
IL-2 Receptor (High Affinity)
Antigen-MHC II/T-cell Receptor
IL-2
T-cell proliferation feedback loop

Figure 2-4: Tri-site anti-rejection therapy in the Edmonton Protocol.
The combination of induction anti-IL2 receptor antibody with maintenance sirolimus and low dose tacrolimus effectively prevents allograft rejection and controls autoimmune recurrence (Adapted form A.M.J. Shapiro, PhD Thesis 2001, with kind permission).

immunosuppressive protocols are still needed to improve safety, reduce diabetogenicity, and ultimately facilitate tolerance induction. The following chapter provides a detailed review of immunosuppressive strategies in clinical islet transplantation, with an emphasis on key agents that have demonstrated considerable potential in promoting tolerance.

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CHAPTER THREE

ISLET CELL TRANSPLANTATION IN PATIENTS WITH DIABETES MELLITUS: CHOICE OF IMMUNOSUPPRESSION

Sulaiman A. Nanji[1] and A.M. James Shapiro[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada

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NOTE: A modified version of this chapter is in press in Biodrugs.

3.1 EARLY IMMUNOSUPPRESSIVE STRATEGIES IN ISLET TRANSPLANTATION

In the early era of clinical islet transplantation, the majority of transplants were combined islet-kidney allografts, since initiating immunosuppression in islet-alone recipients was felt to be inappropriate at that time. The choice of anti-rejection therapy was based largely on the successful outcomes of therapies in solid organ transplantation. Therefore, the mainstay therapy for the vast majority of the greater than 450 islet transplants performed prior to the Edmonton Protocol consisted of azathioprine, cyclosporine, and glucocorticoids.

3.1.1 AZATHIOPRINE

Azathioprine was used initially as an adjunctive immunosuppression to potentiate the effects of cyclosporine and steroids. Interestingly, azathioprine remains one of the few immunosuppressive agents that do not appear to have an adverse effect on beta-cell function or on insulin sensitivity when used alone. Furthermore, its benefit in previous islet transplant protocols may have been to reduce the amount of steroid required.

3.1.2 CYCLOSPORINE

The potent immunosuppressive properties of cyclosporine (CsA) were discovered in 1976 (1), and its introduction into clinical practice by Calne and colleagues in 1979 (2) revolutionized outcomes in solid organ transplantation. CsA blocks the clonal expansion of resting T cells by inhibiting the transcription of genes encoding interleukin-2 (IL-2) and the high-affinity IL-2 receptor, which are essential for T cell activation (3-5) (Figure 3-1). The most important toxic side effects of cyclosporine therapy in islet

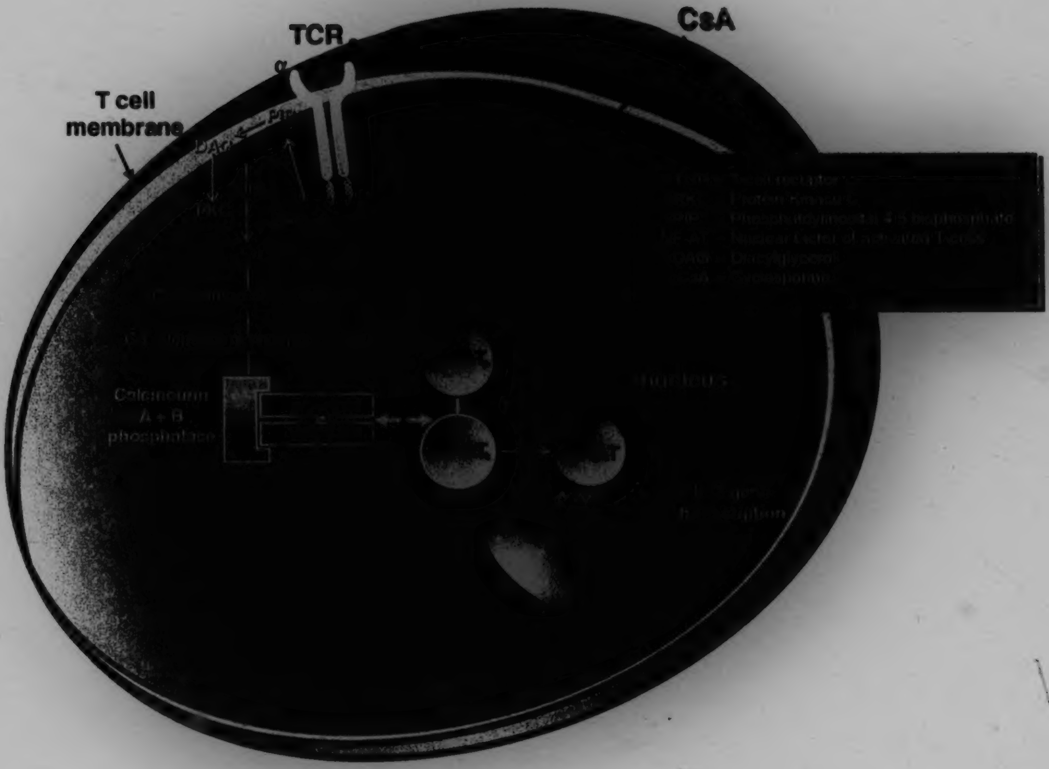
TCR
CsA
α
T cell membrane
DAG
PIP2
TCR = T cell receptor
PKC = Protein Kinase C
PIP2 = Phosphatidylinositol 4,5 bisphosphate
NF-AT = Nuclear factor of activated T cells
DAG = Diacylglycerol
CsA = Cyclosporine
Calcineurin A + B phosphatase
nucleus

Figure 3-1: Intracellular mechanisms of action of the drug cyclosporine.
(Adapted from Immunology, 2nd Edition, Kuby, J. 1994, p.286)

transplantation are nephrotoxicity, since many diabetic patients have underlying renal disease, and diabetogenicity. The diabetogenic potential of CsA was initially reported in 1984 (6), based on a series of human pancreas/kidney transplant recipients converted from azathioprine and prednisone to CsA and prednisone. The underlying impairment in glucose homeostasis was thought to be mediated by peripheral insulin resistance. Subsequent in vitro studies of mouse (7), rat (8) and human islets (9) exposed to high doses of CsA revealed harmful effects on β-cell function as demonstrated by decreased glucose-stimulated islet insulin synthesis or reduced islet insulin content. Ensuing in vivo studies showed that oral administration of CsA to normal rats could induce reversible hyperglycemia and hypoinsulinemia (10, 11). Histological evaluation of the pancreas in CsA treated rats revealed β-cell degranulation and vacuolization, and isolated islets from these animals had a 50% reduction in mRNA synthesis (12). Studies in dogs also revealed progressive, reversible, dose-dependent impairment of insulin secretion in normal dogs given CsA therapy (13-15).

Large animal and human data regarding toxic effects of CsA on β-cells is often difficult to interpret due to confounding factors such as wide variations in dosing that reflect different practices, as well as the relative contributions of other agents in a multi-drug regimen of CsA, azathioprine and steroids. In patients receiving CsA as part of triple immunosuppression with steroids and azathioprine, the incidence of post transplant diabetes varies between 4% and 20%, and of these 40% will require insulin therapy (16, 17). In a study examining the incidence of post-transplant diabetes in renal transplant recipients, patients randomized to treatment with CsA, prednisone and azathioprine had an higher rate of diabetes compared with patients receiving only azathioprine and prednisone, despite a reduction in steroid dose (18). Another study showed defective β-cell function by arginine-potentiated glucose stimulation in psoriasis patients treated with CsA, but not in arthritis patients receiving long-term steroids, suggesting that CsA was responsible for the impaired β-cell function. In the same study, pancreas transplant recipients treated with CsA, prednisone and azathioprine had significantly less insulin secretion compared to control subjects (19).

3.1.3 CORTICOSTEROIDS

Like CsA, corticosteroids are potent immunosuppressive agents that have been a cornerstone in effective anti-rejection therapy. Since corticosteroids act at multiple sites, and essentially suppress the entire immune system, they are associated with several side effects including steroid-induced diabetes. The underlying mechanisms leading to the development of diabetes in patients treated with steroids are multifactorial and include a reduction in insulin sensitivity, down-regulation of insulin receptors, reduced insulin receptor affinity, impairment of post-receptor signaling, reduced peripheral glucose uptake, and altered glucose/free fatty acid cycle kinetics (16, 20-23). The diabetogenic consequence of steroids is likely to be potentiated by cyclosporine, since both are cleared by cytochrome P-450 metabolism and it has been shown that prednisone clearance was significantly lower in renal transplant recipients treated with cyclosporine and steroids, compared with azathioprine and steroids (24, 25).

Given the diabetogenic effects of glucocorticoid therapy, avoidance of these immunosuppressive agents in islet transplantation is critically important. The first clinical trial of steroid avoidance in islet transplantation was reported by Ricordi et al. (26) in a series of 22 patients undergoing cluster islet-liver allotransplantation after abdominal exenteration for malignancy. In most cases, the islets were isolated from a single multi-visceral donor pancreas and embolized into the liver via the portal vein. More than half of the recipients were able to achieve and maintain insulin independence before succumbing to recurrent malignancy. The main factors thought to contribute to the high rate of insulin independence in this trial were the absence of an autoimmune background and the use of steroid-free immunosuppression, where high-dose tacrolimus was used as monotherapy.

Perhaps the most hopeful data prior to the development of the Edmonton Protocol came from the University of Milan regarding their experience from two immunosuppressant protocols in type 1 diabetic islet after kidney recipients (27). In the first Era (1989-1996) the immunosuppressant protocol consisted of anti-lymphocyte serum (ALS) + cyclosporine + azathioprine + prednisone, whereas in the second Era (1998-2001) it consisted of anti-thymocyte globulin (ATG) + cyclosporine + mycophenolate + metformin together with anti-oxidants. Although rejection rates were very low in both eras (3/21 in Era one vs. 3/20 in Era two), the rate of insulin independence was enhanced from 33% to 59% with the elimination of prednisone and addition of mycophenolate and metformin. More than half of patients in the steroid-free protocol maintained insulin independence beyond one year, likely due to more effective and less diabetogenic immunosuppression together with improved insulin action.

3.2 EVOLUTION OF IMMUNOSUPPRESSIVE THERAPY: THE EDMONTON PROTOCOL

A new protocol initiated at our institution in 1999 was designed to comprehensively address the limitations in improved outcomes in clinical islet transplantation (Figure 3-2). The most significant obstacles included 1) Inadequate islet transplant mass, 2) Inadequate islet potency, and 3) use of toxic and diabetogenic immunosuppression. In order to improve islet mass, patients in the Edmonton Protocol received islets prepared from an average of 2 donors corresponding to an average of 850,000 islets. Islet function was optimized by timely processing of the graft, using a

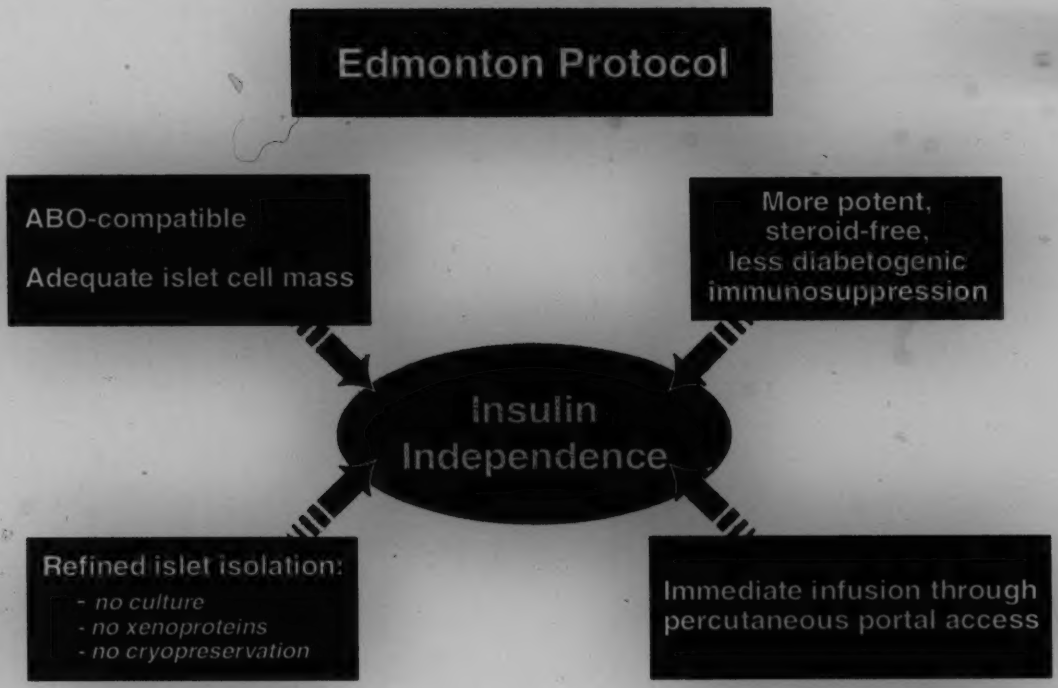
Edmonton Protocol
ABO-compatible
Adequate islet cell mass
More potent, steroid-free, less diabetogenic immunosuppression
Insulin Independence
Refined islet isolation:
- no culture
- no xenoproteins
- no cryopreservation
Immediate infusion through percutaneous portal access

Figure 3-2: Summary of key principles in the Edmonton Protocol.

purified low-endotoxin collagenase enzyme for islet isolation, avoiding exposure to xenoproteins (fetal calf serum) in culture, and immediately transplanting islets into the liver via the portal vein. Less diabetogenic and effective, steroid-free immunosuppression was achieved with the combination of sirolimus with low dose tacrolimus. An inductive course of daclizumab, an anti-IL-2 mAb, was also included to replace steroids, which further reduced the diabetogenic impact of the therapy. All of the initial seven patients treated with the Edmonton Protocol were consistently able to achieve and maintain insulin independence (28).

3.2.1 *SIROLIMUS AND TACROLIMUS*

The discovery of sirolimus and its accelerated implementation into clinical practice has been a very significant contribution to clinical transplantation in the recent decade. With its potent immunosuppressive properties, lower rejection rates, and acceptable toxicity profile, sirolimus has considerable advantages over calcineurin inhibitors particularly for islet transplantation. Sirolimus blocks T and B lymphocyte responses to IL-2 and other cytokines by interference with phosphorylation events that would otherwise follow binding of IL-2 to its receptor. By impeding cytokine action, T and B cell recruitment, activation and expansion are prevented. The actions of sirolimus are more specific than other anti-proliferative agents, since the drug prevents only growth factor induced mitogenesis, leaving other proliferative pathways intact (29, 30) (Figure 3-3).

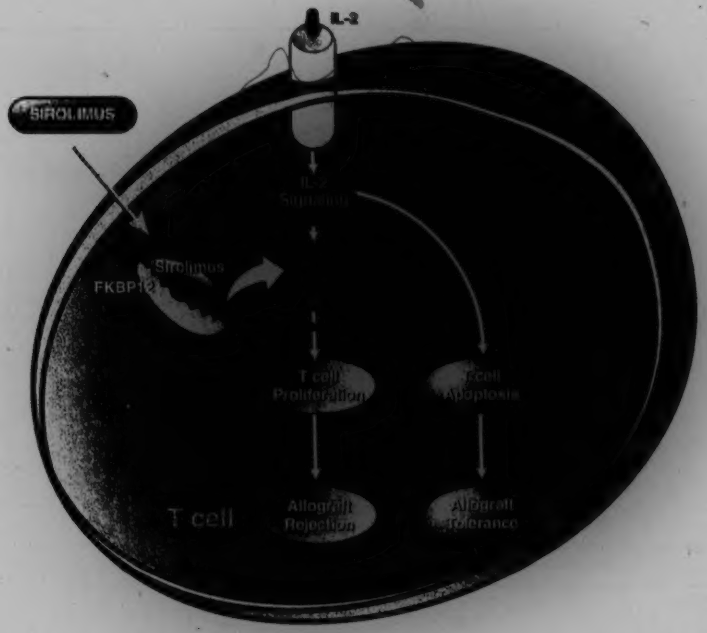
IL-2
SIROLIMUS
IL-2
Signaling
Sirolimus
FKBP12
T cell
Proliferation
T cell
Apoptosis
T cell
Allograft
Rejection
Allograft
Tolerance

Figure 3-3: Intracellular mechanisms of action of the drug sirolimus (rapamycin). By effectively blocking T cell proliferation without compromising IL-2 mediated T cell deletion, sirolimus can prevent allograft rejection while also facilitating allograft tolerance.

Tacrolimus (FK506) is a macrolide antibiotic that was first explored for its potent immunosuppressive properties in 1987 (24, 31). While structurally unrelated to cyclosporine, it shares many of the same intra-cytoplasmic pathways to inhibit the action of calcineurin with subsequent blockade of IL-2 production (Figure 3-4). Although similar in mechanisms of action, tacrolimus has been shown to be 10-100 times more potent than CsA in vitro by inhibition of mixed lymphocyte culture and the generation of cytotoxic T cells (32).

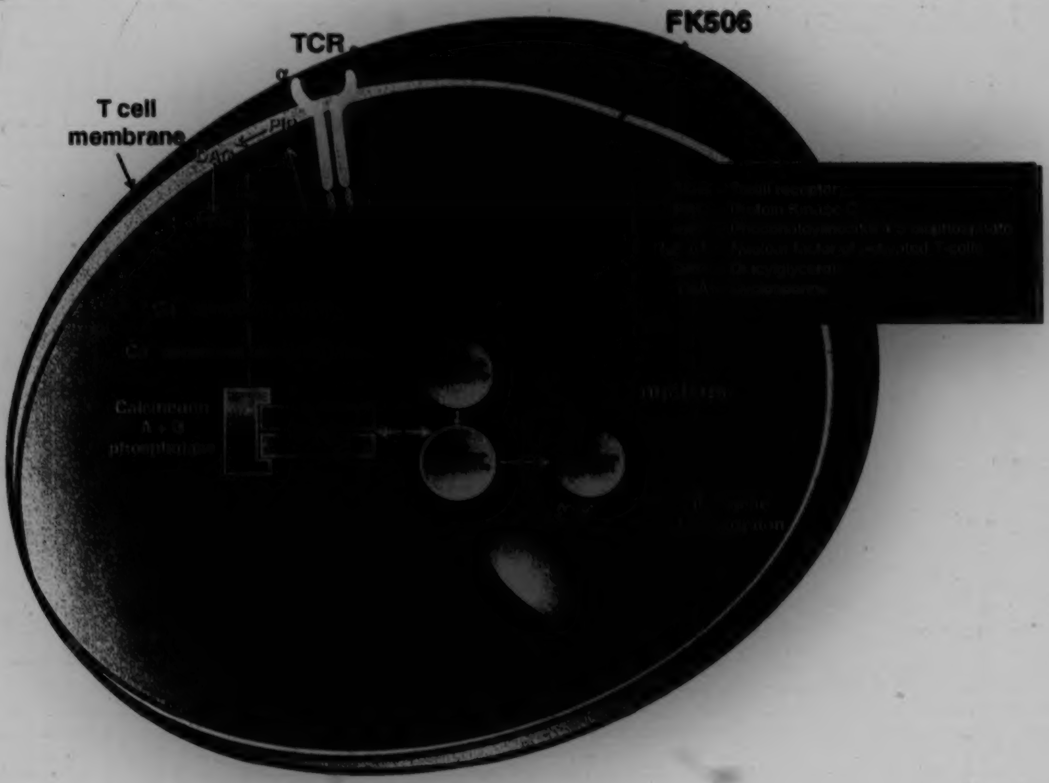

Figure 3-4: Intracellular mechanisms of action of the drug tacrolimus (FK506).

The first use of tacrolimus in clinical transplantation was by Starzl and colleagues who demonstrated that patients with a liver allograft with refractory rejection could be rescued by conversion from CsA to tacrolimus (33). A single-centre randomized trial of tacrolimus vs. CsA in liver transplantation showed a lower incidence of acute rejection, and a reduced need for steroids in patients treated with tacrolimus (34). Other large multi-centre randomized trials of tacrolimus vs. CsA in liver transplantation clearly demonstrated a greater benefit with tacrolimus, with significantly enhanced liver graft and patient survivals compared with cyclosporine (35, 36). One of the first uses of tacrolimus in islet transplantation was in the aforementioned study on non-diabetic patients undergoing combined islet-liver transplantation after abdominal exenteration (26). These patients received high-dose tacrolimus as monotherapy, leading to insulin independence in 55% of patients for periods ranging from 5-58 months post transplant; graft loss was generally due to death from recurrent malignancy (37). The use of tacrolimus in combination with glucocorticoids only rarely has resulted in even temporary insulin independence in type 1 diabetic patients undergoing islet transplantation (38, 39).

The concept of combining sirolimus with calcineurin inhibition in islet transplantation is supported by studies demonstrating their synergistic potential. Prolongation of canine islet allograft survival was seen when sirolimus was given in combination with sub-therapeutic CsA, whereas either drug given alone did not facilitate survival (40). No significant hepatic or renal toxicity was noted, and there was no evidence of deranged glucose homeostasis. A further study in pigs demonstrated that temporary sirolimus and CsA treatment combined with desferoxamine prevented islet allograft rejection (41). Further studies aimed at specifically examining the effect of combined sirolimus and tacrolimus therapy have clearly demonstrated strong synergy between these two agents. Although in vitro studies had originally suggested that sirolimus and tacrolimus could not be given in combination since they both bind to identical cytosolic binding proteins (42), these concerns were not realized upon further investigation. Studies in mice demonstrated that this interactive effect does not occur in vivo due to the abundance of FKBP binding sites that cannot be saturated at physiological dose levels, and that there is in fact synergy when both drugs when used in combination (43, 44). This was subsequently confirmed in a primate renal allograft transplant model where synergistic prolongation of renal allograft function was observed in the combined low-dose tacrolimus + sirolimus treated group (45). McAlister and colleagues used the combination of low-dose tacrolimus with sirolimus and glucocorticoids in an initial series of 32 liver, kidney and pancreas transplant recipients, and found evidence of acute rejection in less than 5% of cases – an unprecedented low rate of rejection in any previous clinical transplant experience (46). These findings clearly had promising implications for the role of sirolimus combined with tacrolimus for clinical islet transplantation.

The drug sirolimus is also highly effective in suppressing inflammatory responses and neointimal hyperplasia in cardiac allografts, and its local impact in preventing atheromatous deposition has been explored successfully in coronary arterial stents (47). What is unclear presently is whether sirolimus impairs neovascularization of islet grafts. Perhaps even more importantly, sirolimus may impair proliferative responses and repair processes within an islet graft, and may interfere with β-cell regeneration from stem cells. Further studies are underway to more fully characterize these concerns.

3.2.2 ANTI-INTERLEUKIN-2 RECEPTOR MONOCLONAL ANTIBODY

One of the most important advances in the Edmonton immunosuppressive protocol was the complete avoidance of glucocorticoids. Immunosuppressive efficacy was maintained with an induction course of humanized anti-IL-2R mAb, also known as anti-CD25 mAb. Extensive trials have demonstrated efficacy of monoclonal antibodies directed against the IL-2 receptor alpha chain for induction therapy in transplantation. In the resting state, only the beta and gamma chains of the IL-2 receptor are expressed on T cells. In the activated state, the alpha chain (CD25) becomes expressed (48). Since the IL-2 receptor alpha chain is expressed only by activated lymphocytes, this provides more specific and targeted immunosuppression. By binding to the exposed alpha chain, anti-CD25 mAb prevents downstream phosphorylation of STAT5 (48).

Initial clinical trials used a rodent antibody to IL-2R, and were as effective but better tolerated than anti-thymocyte globulin, permitting lower target levels of cyclosporine to be given (49, 50). However, the development of antibodies to this agent and short plasma half-life limited treatment to only a few days after transplant. Chimeric and humanized versions of the anti-IL-2R mAb were subsequently developed, with only the original antibody binding sites being of rodent origin, and the remaining portions being human. Two antibody preparations, basiliximab (chimeric) and daclizumab (humanized), have been evaluated in phase III clinical trials (51, 52). In both studies, therapy was compared to placebo with maintenance immunosuppression consisting of cyclosporine and glucocorticoids ± azathioprine. In both trials, the antibodies were well tolerated without cytokine release phenomena, and reduced the incidence of acute rejection by approximately 35% without any increase in infectious or malignancy-related complications. These results were maintained at one-year post transplant, with 27% acute rejection rates in the daclizumab arm compared with 47% in the placebo control group (53). Another large multicenter trial comparing a two-dose daclizumab regimen with no antibody induction in whole pancreas transplantation demonstrated lower acute rejection rates and improved graft outcome, without associated increase in infectious or malignancy-related complications (54, 55).

The risk of post transplant lymphoproliferative disorder (PTLD) is much lower than seen with previous T-cell depletional therapies such as OKT3. Indeed, a cohort of patients was given repeated monthly courses of daclizumab for over a year for intractable psoriasis, and the treatment was efficacious and without detrimental side effects (56). There is no evidence to suggest that anti-IL-2R mAb treatment would be damaging to islet function, although this has not been formally tested. The addition of short term anti-CD25 mAb in the Edmonton Protocol offered the potential to: a) spare steroid use, b) minimize dependence on high dose diabetogenic calcineurin inhibitor therapy, and c) further preserve islet allograft function by reducing potential risk of acute rejection.

3.3 FUTURE DIRECTIONS IN IMMUNOSUPPRESSION: TOWARDS TOLERANCE

The possibility of achieving a permanent state of unresponsiveness to an allograft without the need for chronic immunosuppression remains an important focus in transplantation research. However, attainment of a tolerant state is not the only presiding factor limiting the rapid, broader application of islet transplantation in the earliest stages of diabetes. If the risk of chronic long-term immunosuppression could be substantially reduced by a dramatic reduction in degree of systemic immunosuppression, this would significantly accelerate progress towards the ultimate goal. It has been suggested that islet transplantation could serve as a primary testing ground for novel tolerance protocols since a lack of efficacy would result in the patient's return to insulin therapy rather than potential death in the case of failure of a life-sustaining heart or liver transplant. Moreover, the fact that islets can survive in culture provides the opportunity to not only to immunologically manipulate the graft during the cultured state but also optimize recipient conditioning prior to transplantation.

Although islet transplantation offers a unique opportunity to test new tolerance strategies for clinical application, it may prove to be a challenging model because of the need to overcome both alloimmune and autoimmune barriers, and different mechanistic approaches may ultimately be required to achieve this. At present, all tolerance strategies involve the use of immunosuppression for a limited period of time in order to induce donor unresponsiveness. In recent years, there has been a growing number of agents that have been shown to promote tolerance; these can be broadly classified as agents that act to deplete lymphocytes, interfere with signaling events required for lymphocyte activation, or alter trafficking and recruitment of lymphocytes required for allograft rejection (figure 3-5).

3.3.1 DEPLETIONAL T CELLS AGENTS

The depletion of T cells at the time of transplantation is a critical aspect in several tolerance protocols because of the need to contend with the high frequency of allo-reactive T cells. In recent years, anti-CD3 induction therapy has demonstrated considerable promise in facilitating a state of donor unresponsivess through profound depletion of T cells. An anti-CD3 diphtheria-based immunotoxin (IT) has been shown to promote tolerance in several studies of nonhuman primate renal

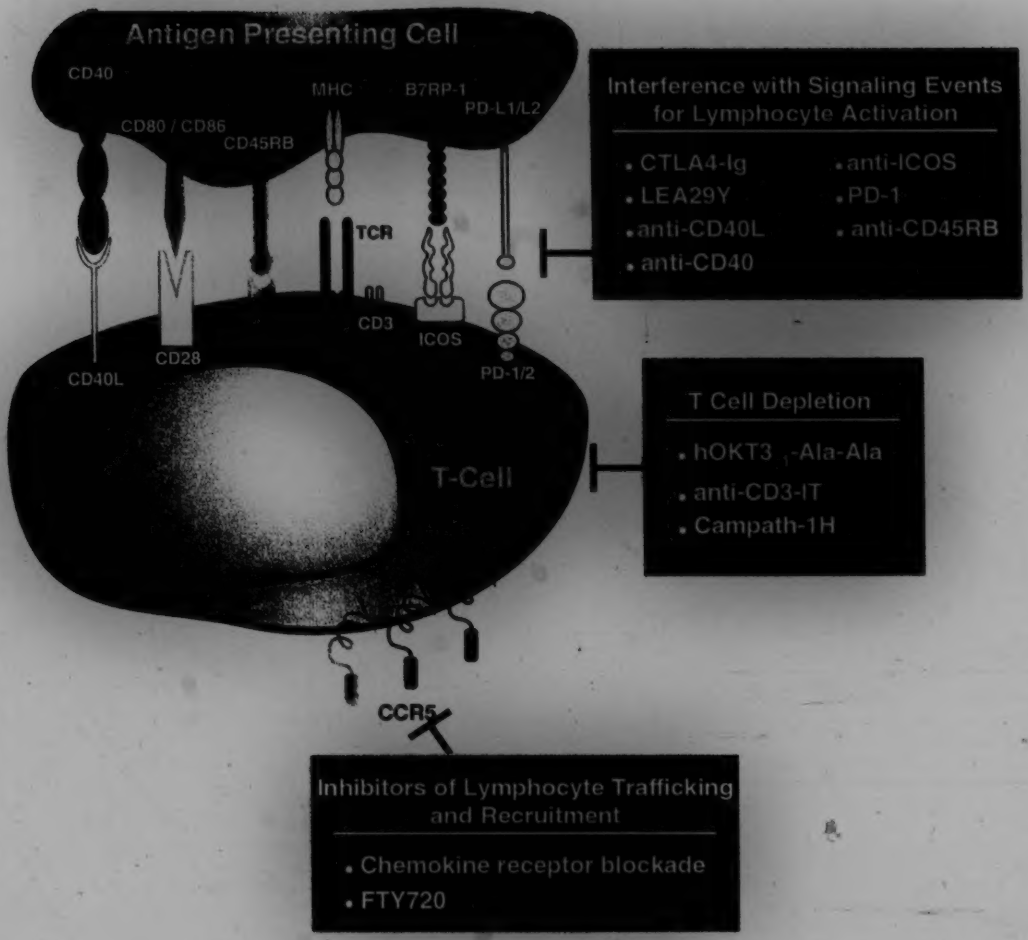
Antigen Presenting Cell
CD40
MHC
B7RP-1
PD-L1/L2
CD80 / CD86
CD45RB
TCR
CD3
ICOS
PD-1/2
CD28
CD40L
T-Cell
CCR5
Interference with Signaling Events for Lymphocyte Activation
CTLA4-Ig
LEA29Y
anti-CD40L
anti-CD40
anti-ICOS
PD-1
anti-CD45RB
T Cell Depletion
hOKT3 $_1$-Ala-Ala
anti-CD3-IT
Campath-1H
Inhibitors of Lymphocyte Trafficking and Recruitment
Chemokine receptor blockade
FTY720

Figure 3-5: Novel agents with the potential of promoting tolerance in islet transplantation and their points of action.

transplantation, either alone (57) or in combination with 15-deoxyspergualin (DSG) (58-60). In islet transplantation, Thomas et al. have demonstrated long-term concordant xenograft survival in primates with spontaneous insulin dependent diabetes with a protocol consisting of IT and cyclosporine (60, 61). The same group subsequently demonstrated that the combination IT + DSG during the peri-transplant period could induce a state of operational tolerance in streptozotocin-treated diabetic primates receiving single-donor islet infusions (62).

Another promising T cell depleting agent that is of particular interest in islet transplantation is hOKT3γ_1-Ala-Ala in view of its efficacy in controlling autoimmunity. The effectiveness of this agent in autoimmune diabetes was initially demonstrated in non obese diabetic (NOD) mice (63), and was subsequently shown to impede the progression of diabetes and improve metabolic control in children treated at the time of their diagnosis (64). At the University of Minnesota, inductive treatment with hOKT3γ_1-Ala-Ala has been used in a series of islet transplant recipients who have achieved insulin independence with single donor islet infusions (65, 66). This represents a significant development, given that patients treated in the Edmonton Protocol required islets from an average of 2 donors.

Campath-1H (Alemtuzumab), a humanized antibody directed against CD52 determinants on the surface of T-cells, also demonstrates considerable potential given its ability to deplete lymphocytes for prolonged periods of time. While the precise mechanisms of action are not fully understood, it has been shown to prevent T cell activation via CD45 signaling events, and does not interfere with T cell receptor activation (67). This agent has been particularly effective in control of autoimmune diseases, including acute vasculitides (68), multiple sclerosis (69) and in autoimmune cytopenias (70, 71). Calne et al. used Campath-1H for induction prophylaxis followed by only half-dose cyclosporine as maintenance therapy in 31 patients undergoing renal transplantation (72, 73). Remarkably, after a mean two-year follow-up, 28 patients had functioning grafts, with an incidence of acute rejection of 12.9%, and with a rate of infectious complications that did not differ from a control series of standard therapy. Recently, Knechtle et al. reported preliminary results of their pilot study of Campath-1H induction therapy in combination with sirolimus monotherapy in renal transplant patients and found that this strategy is not as effective as the combination of Campath-1H with calcineurin inhibitors (74). Similarly, preliminary data on the combination of Campath-1H and sirolimus in a series of islet transplant recipients at our institution revealed that, in the absence of therapeutic dosing of calcineurin inhibitors, sirolimus and Campth-1H is no more effective than the Edmonton Protocol in preventing allograft rejection. Although the immunosuppressive benefits of Campth-1H are still under investigation, its potential in islet transplantation has yet to be fully defined. Preliminary studies suggest that it may offer effective control of rejection and autoimmunity provided T cell depletion is combined with additional immunomodulatory strategies such as mycophenolate or tacrolimus.

3.3.2 Agents that Interfere With Signaling Events Required For Lymphocyte Activation

Agents aimed at targeting cell surface molecules involved in T cell activation hold tremendous potential in promoting tolerance in patients. One of the most promising strategies involves the blockade of critical co-stimulatory signals necessary for the activation and clonal expansion of T cells. Blocking costimulation while leaving T-cell receptor-antigen engagement unaltered effectively renders the alloreactive T cell population anergic, forcing them to apoptosis (75-77). The CD28:B7 and CD40:CD40L costimulatory pathways play crucial roles in regulating T cell immune responses, and the blockade of these pathways with CTLA4-Ig or CD40L mAb, respectively, has been shown to promote long-term allograft survival in a variety of transplantation models. Specifically, in islet transplantation, the efficacy of these agents in promoting tolerance has been validated in studies in nonhuman primates (78-80). Using a humanized anti-CD40L mAb (hu5C8), Kenyon et al. reported long-term insulin independence in pancreatectomized diabetic recipient monkeys following intra-portal islet transplantation without any toxicity or infectious complications (79, 80). Moreover, Zeng et al. have shown that blockade of CD40L signaling is effective at preventing rejection is a sensitized murine model of islet transplantation, which is of considerable interest in islet transplantation given that multiple donors are usually required to achieve insulin independence (81).

While the blockade of CD40L signaling demonstrated impressive experimental evidence in primate models, Phase I trials with the humanized hu5C8 antibody were terminated due to unexpected thromboembolic complications that resulted in one patient mortality (82, 83). These thromboembolic events have also been observed in primate models, and are likely associated with high levels of CD40L expression both on platelets and on endothelium (84, 85). Since the potential of CD40L blockade was so potent, efforts have recently been refocused on blocking the CD40 epitope on antigen presenting cells, thereby circumventing the T cell and concerns of cross-reactivity with platelet and endothelium altogether (86). Recently, this approach has demonstrated prolongation of renal allografts (87) as well as islet allografts (unpublished observations, Larsen CP et al.) in primates, without evidence of thromboembolic complications. Furthermore, development of LEA29Y, a second generation CTLA4-Ig with approximately 10-fold more potency in vitro has demonstrated significant benefit in primate models of islet transplantation when combined with sirolimus and an anti-IL-2R mAb (88). Presently, a multicenter Phase III clinical trial of LEA29Y is underway in renal transplant recipients, and preliminary results suggest low rates of acute rejection, excellent graft function and minimal side effects.

In recent years novel costimulatory molecules have been identified which may serve as promising immunosuppressive targets in islet transplantation (89). For instance, data from our own laboratory has revealed that the inducible costimulator (ICOS) molecule plays an important role in islet allograft rejection, and blockade of ICOS signaling results in a significant reduction in allospecific T cell proliferation and effector function (90). The role of ICOS blockade in facilitating islet allograft survival has also been demonstrated by others (91). Furthermore, stimulation of negative signals through the novel inhibitory molecule programmed death 1 (PD-1) together with blockade of positive signals through CD40L has demonstrated potent islet allograft survival (92, 93). Finally, CD45 has also demonstrated potential as an immunosuppressive target in islet transplantation. CD45 is a transmembrane protein tyrosine phosphatase involved in the regulation of lymphocyte activation signals. A blocking antibody to the CD45RB isoform has demonstrated considerable success in promoting islet allograft survival in streptozotocin-treated diabetic mice (94-98), and in NOD mice (99, 100).

3.3.3 AGENTS THAT ALTER TRAFFICKING AND RECRUITMENT OF LYMPHOCYTES

The regulation of lymphocyte trafficking and recruitment by interference with chemokine receptors or by treatment with FTY720 has revealed a further promising approach to induce tolerance. Chemokines play a critical role in allograft rejection by virtue of their importance in orchestrating lymphocyte migration and activation. Interference of these pathways through chemokine receptor targeting has shown therapeutic benefit in various experimental models (101, 102). Interestingly, several studies suggest that the expression of a given chemokine receptor system varies in the host rejection response depending on the particular organs or tissue transplanted (103). Hence, each organ or tissue may be dependent on a unique set of chemokines to mediate allograft rejection. In this regard, experimental models based on chemokine receptor knock-out mice or blocking monoclonal antibodies have revealed an important role for CCR2 (104), CCR5 (105), and CXCR3 (106) in islet allograft rejection. Targeting these chemokine pathways may therefore provide a therapeutic strategy to prevent islet allograft rejection in patients.

A less specific approach to inhibit lymphocyte trafficking is the use of the drug FTY720, a novel immunosuppressive agent that interferes with lymphocyte responsiveness to chemokines, causing them to be sequestered into secondary lymphoid organs. An important feature of FTY720 is that it can prevent allograft rejection without inducing generalized immunosuppression; the agent does not inhibit T cell activation or proliferation, cytokine production or B-cell antibody secretion, and does not impair anti-viral memory responses in small animal models (107-109). Phase II clinical trials have been completed in renal transplantation, and demonstrated relative safety apart from lymphopenia and bradycardias (110).

Interest in exploring FTY720 in islet transplantation is based on recent promising data of this compound in experimental and clinical transplantation. It has been shown to prevent allograft rejection in several rodent models of allotransplantation (111-114), and more recently in primate renal transplantation (115). With particular relevance to islet transplantation, FTY720 has been shown to potently inhibit autoimmune diabetes and recurrent disease in NOD mice, as well as enhance insulin action without any diabetogenic side effects in mouse and primate models (116, 117). Moreover, studies at the University of Minnesota and Miami in non-human primate islet transplantation have demonstrated that the combination of FTY720 with RAD (Everolimus) is effective maintenance immunosuppression following basiliximab induction therapy (118). Based on this study, as well as strong preliminary data in mouse models of islet allograft rejection (116) and in autoimmune diabetes (117, 119), clinical trials using FTY720 in islet transplantation are imminent.

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CHAPTER FOUR

A ROLE FOR GRAFT SITE AND LYMPHATICS IN THE EFFECTIVENESS OF COSTIMULATION BLOCKADE-BASED STRATEGIES IN MURINE MODELS OF TRANSPLANTATION

Sulaiman A. Nanji MD[1], Wayne W. Hancock MD, PhD[2], Bin Luo MD[1],
Colleen D. Schur Bsc[1], Rena L. Pawlick Bsc[1], Lin-Fu Zhu MD[1],
Colin C. Anderson PhD[1], A.M. James Shapiro MD, PhD[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta
[2] Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and University of Pennsylvania School of Medicine, Philadelphia, PA

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4.1 INTRODUCTION

Considerable advances have been made in recent years in the development of immunosuppression for organ transplantation, with improved short-term graft survival times and superior patient outcomes (1). Nevertheless, problems of toxicities such as renal dysfunction, diabetes and hypercholesterolemia, and increased risks of malignancies and opportunistic infections associated with chronic immunosuppression remain. Since the first reports of tolerance induction by Medawar and colleagues (2), and Hasek (3) over 50 years ago, extensive efforts have been made to develop clinically applicable strategies to reliably induce life-long, drug-free graft acceptance. Murine models have proven critical to testing novel approaches and providing new insights into the immunobiology of graft rejection.

One promising strategy studied extensively in murine models is the blockade of costimulatory signals. This strategy is based on the principle that two separate but synergistic signals are required for the activation and clonal expansion of naïve T cells. The first signal confers antigen specificity to the immune response and arises from the recognition of foreign antigen in the context of MHC by the T cell receptor. The second signal, known as costimulation, is delivered through the engagement of costimulatory molecules on the surface of the T cell with their ligands on the antigen presenting cell (APC). Since a T cell that perceives antigen in the absence of costimulation becomes anergic and may undergo apoptosis (4-6), blocking costimulation at the time of transplantation may facilitate graft acceptance through inactivation or deletion of alloreactive T cells. While the two best characterized costimulatory pathways are CD28:B7 and CD40:CD154, a series of novel costimulatory molecules have been identified including an inducible costimulator molecule (ICOS) (7). ICOS is structurally similar to CD28 and interaction with its ligand, B7-related protein-1 (B7RP-1), results in the delivery of signals that promote T cell proliferation and differentiation (8, 9).

Various studies have demonstrated that blockade of costimulatory signals, with or without temporary immunosuppressive drugs, can prevent graft rejection in a series of transplant models. However, the rate of successful graft acceptance under these therapeutic conditions can differ depending on the organ or tissue transplanted. It is widely accepted that a hierarchy exists among different allografts in their susceptibility to immune rejection, as well as their response to tolerance promoting protocols (10-12). While skin and small bowel allografts represent the strongest barriers to tolerance induction, liver allografts are often spontaneously accepted across major histocompatability barriers without any immunosuppression, and can even facilitate acceptance of other concomitant donor-strain allografts (13). Between these two ends of the spectrum, pancreatic islets, heart and kidney allografts appear to be progressively more readily accepted in mouse models with or without immunomodulation of the recipient. Several factors such as the vascularity of the allograft, the integrity of lymphatic drainage, the presence of tissue specific antigens and graft size have all been postulated to influence the alloimmune response and hence the outcome of graft acceptance or rejection.

In this study, we show that costimulation blockade of ICOS signaling in combination with cyclosporine (CsA) induces permanent abdominal cardiac allograft survival, but does not facilitate renal subcapsular islet allograft survival. We explored some of the potential factors responsible for the divergent findings in cardiac and islet allograft survival in this model. We provide evidence that it is the characteristics of the graft site rather than properties intrinsic to these different tissues that determines the outcome of transplantation.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

Adult C57BL/6 (H-2^b) mice were used as recipients and fully MHC-mismatched adult and neonatal BALB/c (H-2^d) mice were used as donors. Mice were obtained from Charles River Canada, housed under standard conditions, and cared for in accordance with the guidelines established by the Canadian Council on Animal Care.

4.2.2 ISLET TRANSPLANTATION

C57BL/6 recipient mice were rendered diabetic by an injection of streptozotocin (200 mg/kg i.v., Sigma-Aldrich, Canada). Donor islets were isolated from fully MHC-mismatched BALB/c mice by collagenase digestion (1 mg/ml, Sigma-Aldrich) followed by Ficoll purification (14, 15), and ~500 islets were placed under the left renal capsule of diabetic mice. Successful engraftment was defined by correction of serum glucose level to <8 mmol/L by day 3 post-transplant, and rejection was defined as a rise in serum glucose >15 mmol/L for 2 consecutive days.

4.2.3 CARDIAC TRANSPLANTATION

Heterotopic transplantation of vascularized BALB/c cardiac allografts to C57BL/6 mice was performed as described (16). Briefly, donor hearts were grafted into the abdomen of recipient mice with the ascending aorta and pulmonary artery of the heart graft being anastomosed to the abdominal aorta and inferior vena cava of the recipient, respectively. Rejection was defined as complete cessation of a palpable pulsation, and confirmed at laparotomy. Renal subcapsular cardiac transplantation was performed similarly to the technique of kidney subcapsular thymus implantation (17). Briefly, neonatal BALB/c hearts were harvested and implanted beneath the renal capsules of C57BL/6 mice. Recipients underwent nephrectomy of graft-bearing kidneys at days 8, 10, 12 and 14 post-transplant and histologic analysis of the cardiac grafts was performed.

4.2.4 RENAL LYMPH NODE RESECTION AND SPLENECTOMY

At islet transplantation, recipients underwent microsurgical resection of the local kidney lymph node, located at the junction of the renal artery and abdominal aorta. A splenectomy was also performed by ligating the splenic artery and vein with suture, followed by excision of the spleen.

4.2.5 REAGENTS AND TREATMENT PROTOCOLS

Production and characterization of the non-depleting anti-ICOS mAb (12A8) was described previously (18). CsA was purchased from the University of Alberta pharmacy. Transplanted mice were treated with anti-ICOS mAb (0.1 mg/d) and CsA (10 mg/kg/d) i.p. for 14 days, beginning on the day of transplantation.

4.2.6 PATHOLOGY

Subcapsular grafts (hearts, islets) were harvested at serial intervals or >100 days post-transplant. Samples were subsequently fixed in formalin, paraffin-embedded, and paraffin sections were stained with hematoxylin and eosin to assess overall cellularity of each allograft.

4.2.7 STATISTICAL ANALYSIS

Graft survival was analyzed using the Kaplan-Meier method, and statistical comparisons among groups were performed using the log rank test (SPSS version 10.0 for Macintosh, Chicago, IL).

4.3 RESULTS

4.3.1 ANTI-ICOS AND CSA FACILITATE LONG-TERM CARDIAC BUT NOT ISLET ALLOGRAFT SURVIVAL

In a fully MHC-mismatched strain combination, we compared the effect of ICOS signaling blockade in combination with cyclosporine on cardiac versus islet allograft survival. Recipient mice with either a vascularized cardiac allograft or a renal subcapsular islet allograft were treated with combination anti-ICOS mAb and CsA for 14 days and monitored for graft rejection. All mice receiving cardiac allografts under combination therapy accepted their grafts long-term (>100 days), as compared to control mice that rejected their graft at a median of 13 days (Figure 4-1A). In contrast to cardiac allograft acceptance, recipients of islet allografts that were treated with combination therapy rejected their grafts within 26 days of transplantation, with a median survival of 15 days, compared to 13 days for control mice ($p<0.05$) (Figure 4-1B). Therefore, while combination therapy with anti-ICOS mAb and CsA has a dramatic effect on cardiac allograft acceptance, this therapy only delivers a marginal benefit to islet allograft survival.

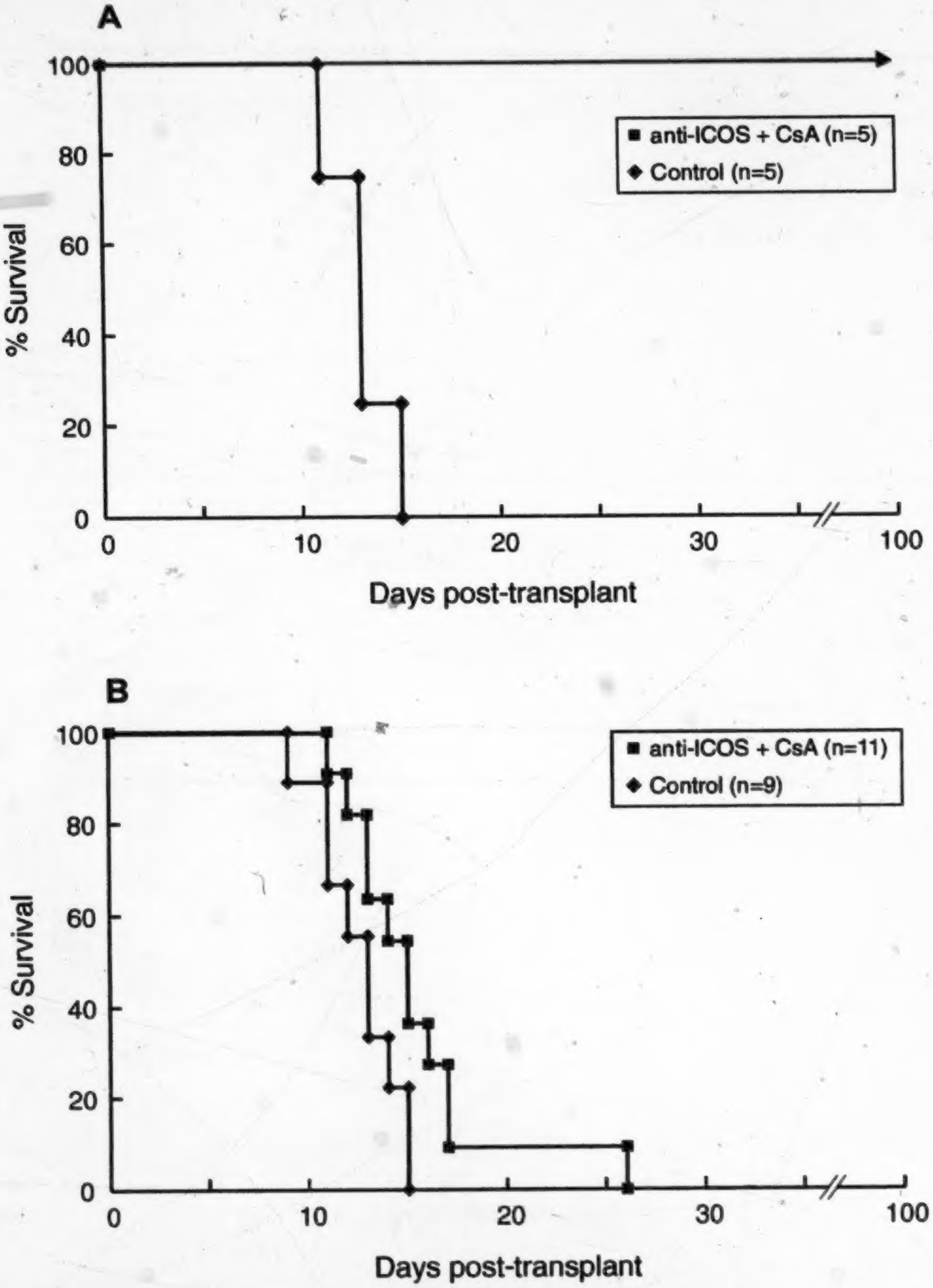


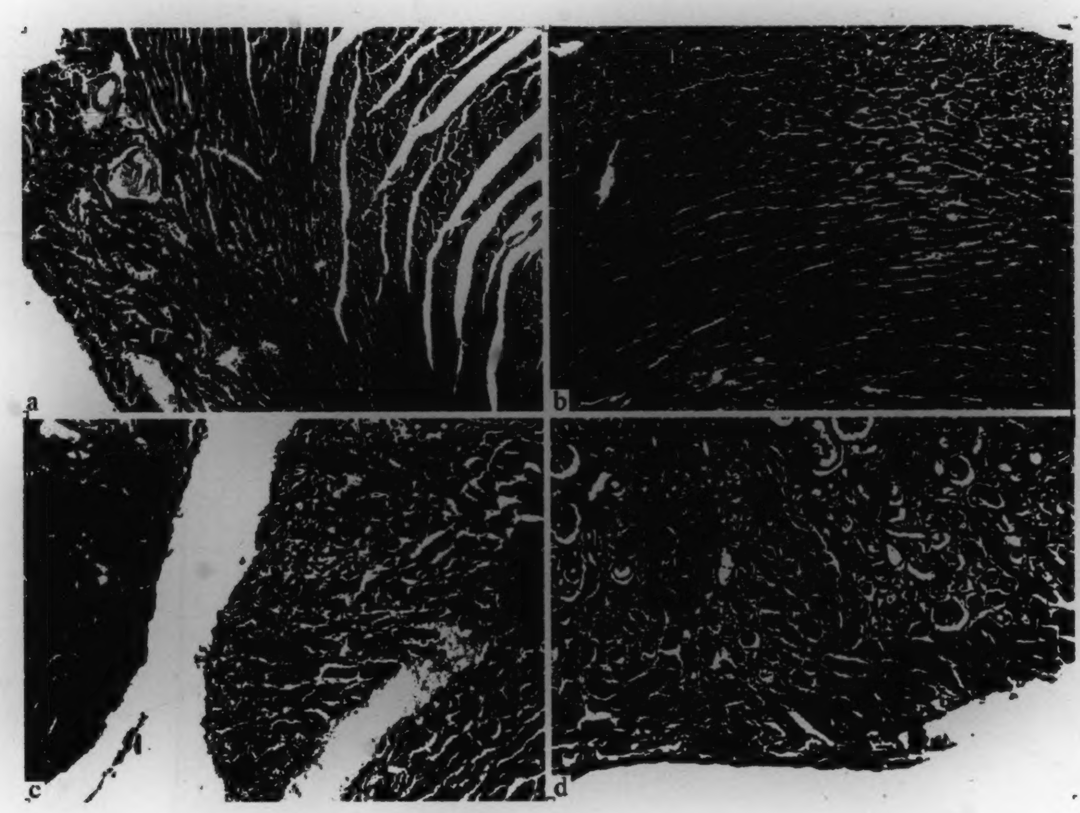
Figure 4-1: Allograft survival under treatment with anti-ICOS mAb and cyclosporine. (A) Heterotopic abdominal cardiac allografts survive indefinitely after treatment with anti-ICOS mAb and CsA, compared to untreated control mice who reject their grafts at a median of 13 days. (B) In contrast to cardiac allograft acceptance, treated recipients of renal subcapsular islet allografts demonstrated only a modest benefit in graft survival compared to control mice (MST=15 versus MST=13, $p<0.05$).

4.3.2 DIVERGENT ALLOGRAFT SURVIVAL MAY RELATE TO THE TRANSPLANT SITE RATHER THAN THE TISSUE BEING GRAFTED

Several factors may account for the divergent findings between cardiac and islet allograft survival in this model. One possibility is intrinsic differences between these tissues. For example, vascularized cardiac allografts contain a large number of endothelial cells, while there is a paucity of such cells in the islet grafts. Another possible explanation may be the difference in the site of implantation. This latter possibility predicts that the same type of tissue would be treated differently by the immune system depending on the location of the transplant.

To evaluate the relative importance of intrinsic tissue differences versus site of transplantation as the major determining factor on allograft survival in our model, we transplanted neonatal cardiac allografts under the renal capsule of recipient mice, and compared these recipients to a cohort receiving adult abdominal cardiac allografts. By doing so, we could compare rejection of essentially homologous tissue when placed in the two different sites employed in this model. If the disparity in outcomes between islet and cardiac allografts is based on inherent tissue differences and not on the site of transplantation, then cardiac allografts should experience the same fate irrespective of the location of transplantation. Neonatal donors were used for the subcapsular cardiac grafts in order that an intact heart could be transplanted and because we found that implantation of pieces of adult heart tissue resulted in technical failures when placed under the renal capsule. After transplantation, mice were either treated with combination anti-ICOS mAb and CsA or left untreated as controls. During the course of treatment mice underwent nephrectomy of their graft-bearing kidneys at scheduled days post-transplantation for histologic evaluation of their cardiac allografts. Interestingly, as early as 8 days post-transplant, subcapsular grafts from treated and control recipients were not beating at the time of harvest, and histologic evaluation revealed marked lymphocytic infiltration resembling acute allograft rejection with focal areas of necrosis. This observation was in stark contrast to the well preserved myocardium in both abdominal allografts from treated recipients, as well as subcapsular allografts from immunodeficient C57BL/6-RAG-KO recipients (Figure 4-2). The fact that renal subcapsular cardiac allografts are rejected while abdominal cardiac allografts are accepted under combined anti-ICOS mAb and CsA therapy suggests that the site of transplantation influences the alloimmune response, and that factors intrinsic to the tissue, such as the presence of endothelium, do not promote long-term survival of cardiac allografts.

A



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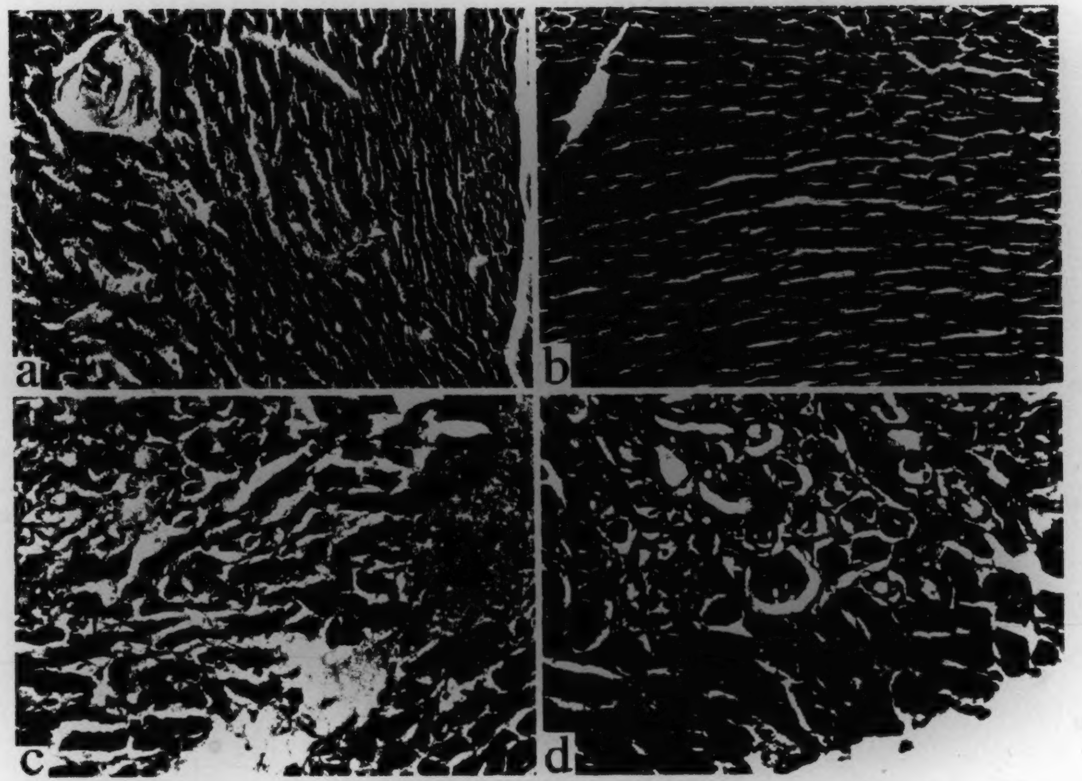

Figure 4-2: Histology of cardiac allografts (BALB/c to C57BL/6)
a) normal adult cardiac tissue; b) abdominal adult cardiac allograft at day 7 post transplant in recipient treated with anti-ICOS + CsA demonstrating negligible mononuclear cell infiltrate and well preserved myocardium; c) rejected subcapsular neonatal cardiac allograft in treated recipient at day 8 post transplant, asterisk indicates focal areas of necrosis; d) well preserved subcapsular neonatal cardiac allograft at day 8 post transplant from immunodeficient recipient. Magnification x150 (A), x300 (B).

4.3.3 INTACT SECONDARY LYMPHOID TISSUE INCREASES THE LIKELIHOOD OF REJECTION OF SUBCAPSULAR GRAFTS

Having established that the graft site influences the outcome of anti-ICOS mAb and CsA therapy, we investigated aspects of the graft site which might contribute to this effect. It is widely accepted that secondary lymphoid organs provide the necessary environment for critical cellular interactions between APC and naïve T cells to initiate immune responses to microbial or allo-antigens (19). Therefore, we hypothesized that renal subcapsular allografts are more susceptible to rejection than abdominal cardiac allografts due to the presence of intact secondary lymphoid tissue. To determine if the absence of secondary lymphoid tissue could facilitate prolongation of graft survival under our treatment protocol, we transplanted islet allografts under the kidney capsule of streptozotocin-treated diabetic mice that had undergone renal lymph node resection and splenectomy. Mice either received anti-ICOS mAb and CsA therapy or were untreated.

Compared to untreated mice maintaining normal lymphatic drainage, untreated mice undergoing splenectomy and renal lymph node resection at the time of islet transplantation demonstrated only a small though significant improvement in islet allograft survival (median survival = 13 days and 17 days, respectively, $p<0.005$) (Figure 4-3A). A more impressive difference was observed when treated recipient mice with intact lymphatic drainage were compared to treated splenectomized and renal lymph node resected recipients; the latter showed significantly longer allograft survival, with ~30% (2/7) of mice demonstrating permanent allograft acceptance (median survival = 15 days and 22 days, respectively, $p<0.005$ (Figure 4-3B).

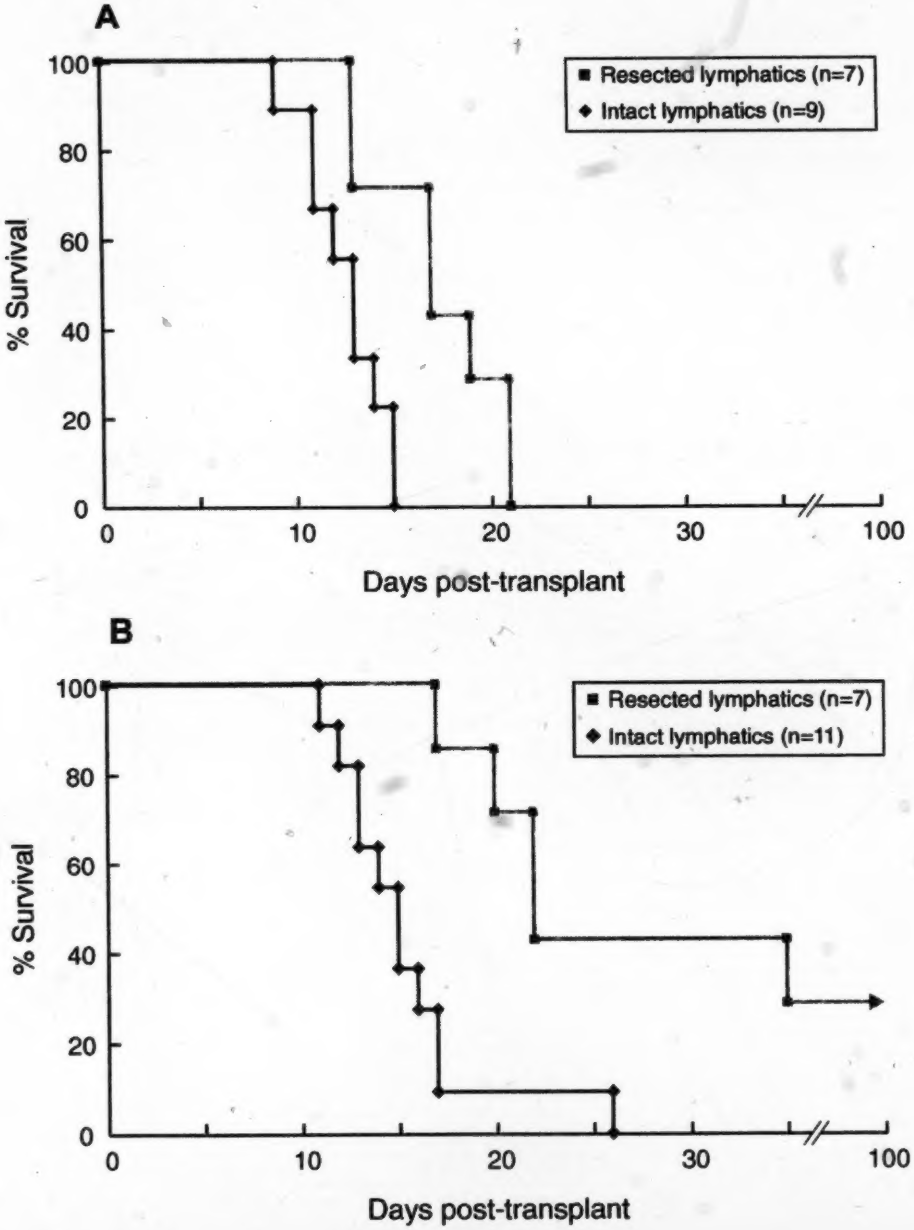

Figure 4-3: The role of lymphoid tissue in facilitating rejection of subcapsular grafts. (A) Mice undergoing splenectomy and renal lymph node resection at the time of transplantation without combination therapy had a significant improvement in allograft survival compared to mice with intact lymphatic drainage, however no mice achieved long-term graft acceptance (MST=17, versus MST=13, $p<0.005$). (B) Splenectomy and renal lymph node resection under conditions of anti-ICOS mAb + CsA therapy led to a more impressive improvement in allograft survival, with ~30% of recipients achieving long-term allograft acceptance compared to treated recipients maintaining intact lymphatic drainage (MST=22 versus MST=15, $p<0.005$).

Histology of islet allografts that were accepted indefinitely in mice having undergone splenectomy and renal lymph node resection demonstrated intact islet tissue with minimal mononuclear cell infiltrate (Figure 4-4). These data suggest that the presence of secondary lymphoid tissues (spleen and lymph node) plays an important role in reinforcing the alloimmune response to renal subcapsular allografts. More importantly, the dependence of rejection on secondary lymphoid tissue is increased under conditions of anti-ICOS mAb and CsA treatment, suggesting that secondary lymphoid tissues may impair the effectiveness of costimulation blockade-based strategies in enhancing graft survival.

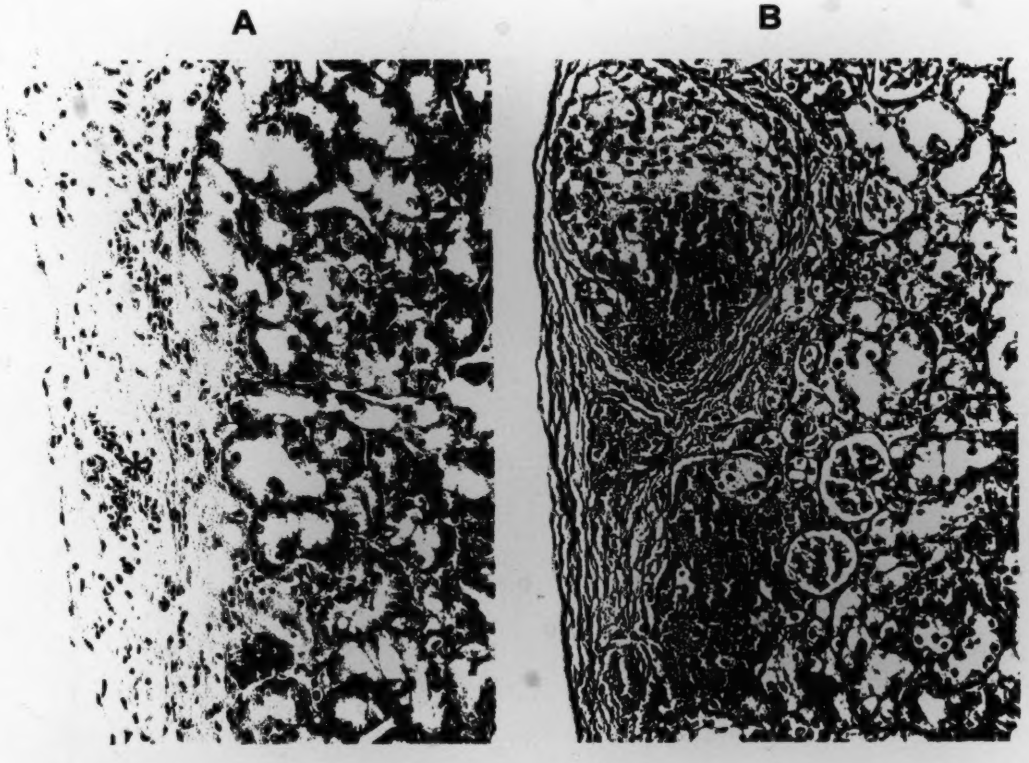

Figure 4-4: Histology of subcapsular islet allografts.
Long-term accepted islet grafts from renal lymph node resected and splenectomized mice treated with combination therapy revealed well preserved islet tissue with negligible mononuclear cell infiltrate (B, day 128 post transplant), compared to rejected allografts from the same cohort of mice (A, day 12 post transplant). Asterisks indicate islet destruction (A) versus preservation (B) (X300 original magnifications, representative of at least 4 grafts/group).

4.4 DISCUSSION

It has long been accepted that the rate of allograft acceptance, as well as the response of an allograft to tolerance protocols, is organ- and tissue-dependent (10-12). While the results of this study further support the concept of a hierarchy among allografts in their response to tolerance induction, they suggest that factors extrinsic to the tissue may contribute to the hierarchy. Using a mAb to block the delivery of ICOS costimulation, in combination with temporary CsA therapy, permanent acceptance of cardiac allografts was achieved in all treated recipients. Previous studies have demonstrated that blockade of ICOS costimulation facilitates cardiac allograft acceptance, either alone or in combination with CsA, with combined treatment leading to indefinite cardiac allograft survival and an absence of transplant arteriosclerosis (18, 20). However, when this therapy was applied to recipients of renal subcapsular islet allografts, graft survival was only marginally prolonged, with all grafts succumbing to rejection within 26 days.

A number of explanations may account for the divergent findings between cardiac and islet allograft survival in this model. One possibility is a difference in the intrinsic cellular composition of the tissues, while another may be due to the difference in location of the allograft, with the renal subcapsular space associated with increased susceptibility to rejection. By grafting heart tissue under the kidney capsule of recipient mice treated with combination anti-ICOS mAb and CsA therapy, we found that in contrast to abdominal cardiac allografts, subcapsular hearts were rejected, suggesting that the site of transplantation may have an important influence on allograft rejection. In this experiment, intact neonatal hearts were implanted beneath the kidney capsule instead of adult cardiac tissue, since the latter failed to engraft under the subcapsular space. While there may be intrinsic differences between these two tissues, the fact that renal subcapsular neonatal hearts are permanently accepted in immunodeficient mice but are rejected readily in competent animals suggests that this is a useful model.

We subsequently explored the role of secondary lymphoid tissue as a potential determinant of graft site on allograft survival. It is widely acknowledged that secondary lymphoid organs such as the spleen, lymph nodes and mucosal lymphoid tissue play a critical role in orchestrating the immune response to an allograft (19). The organized structure of secondary lymphoid organs provides the necessary environment for naïve T cells to undergo activation and differentiation through exposure to alloantigen and costimulatory signals by APCs (21). Experiments in skin allografts reported nearly 40 years ago underlined the importance of lymphatic drainage in the induction of immune responses by demonstrating that rejection and priming responses could not be induced if antigen is prevented from migrating to draining lymph nodes or the spleen (22). Additional evidence from so-called "parking experiments" (23, 24) as well as the study of mice deficient in specific receptors on T cells important in lymph node homing (25) have all further underscored the importance of appropriate antigen-T cell interaction in lymphoid tissue in generating immune responses to allografts. Even vascularized allografts are critically dependent on the presence of host lymphoid organs for rejection, as evidenced by the fact that cardiac allografts are permanently accepted in recipient mice that lack secondary lymphoid tissue (26). More recently, the use of T cell receptor transgenic models has provided a powerful tool to evaluate allospecific T cell responses in vivo, demonstrating that T cell activation occurs initially in the draining lymph nodes followed by the spleen in recipient mice (27). With respect to renal subcapsular islet allografts, this initial activation is likely to occur in the local renal lymph nodes (28).

In this study, vascularized cardiac allografts were transplanted into the recipient abdomen, in the absence of any intact lymphatic drainage to secondary lymphoid organs. Conversely, allogeneic islets were implanted beneath the kidney capsule, where passenger leukocytes could migrate to the local lymph nodes of the recipient kidney. Therefore, we hypothesized that the integrity of lymphatics could be a critical determinant of graft site on allograft survival, and that the increased susceptibility of the renal subcapsular space to rejection is based on the presence of intact lymphatic drainage. By conducting islet allotransplants in diabetic mice that had undergone renal lymph node resection and splenectomy, we demonstrated that the absence of these secondary lymphoid tissues significantly enhanced allograft survival. Although untreated mice without the lymph node and spleen demonstrated a significant improvement in graft survival, all of the islet allografts were rejected by 21 days. In contrast, mice having undergone renal lymph node resection and splenectomy that were treated with anti-ICOS mAb and CsA had a more dramatic improvement is allograft survival, with ~30% of mice permanently accepting their allografts. Long-term graft function in these mice was confirmed by a return to hyperglycemia following nephrectomy of the graft-bearing kidney at >100 days. The more striking improvement in allograft acceptance in treated mice in the absence of secondary lymphoid tissue suggests that these tissues play an even greater role in reinforcing the alloimmune response under conditions of costimulation blockade. The corollary, therefore, is that secondary lymphoid tissues may pose a significant barrier in the effectiveness of costimulation blockade in promoting long-term allograft acceptance.

Although we have demonstrated the importance of lymphatic drainage in the susceptibility of islet allografts to rejection, other basic differences between islets and cardiac tissue may contribute to the differences in the outcomes of graft survival observed. For instance, the mode of vascularization between cardiac and islet allografts may impact on their susceptibility to rejection (11). Nonvascularized islet grafts are exposed to conditions of ischemia with resulting nonspecific inflammation and tissue necrosis, which may in turn facilitate progression to immune destruction. Conversely, vascularized cardiac grafts are relatively spared from the destructive processes of nonspecific inflammation, thereby providing a more favorable environment for graft acceptance. Another important consideration between cardiac and islet grafts that may influence the predisposition of these tissues to rejection is the size of the graft. The concept being that the larger the graft the larger the number of cells that must be destroyed before the graft ceases to function and is deemed rejected. The importance of the mass of donor tissue on graft rejection has been supported by the finding that concurrent implantation of multiple allografts in a single recipient results in long-term acceptance of all the allografts, whereas transplantation of a single allograft results in rejection (29). Based on this observation, the significant disparity between the size of an islet and cardiac graft may also contribute to the differences observed in graft survival in our model.

Another potential contributor to the inferior survival of islet allografts may be the use of CsA in our treatment protocol, given its well-described deleterious effects on islet cells. CsA has been shown to impair beta cell function both in vitro (30, 31) and in vivo (32), prevent islet replication (33) and impede neovascularization of islets transplanted beneath the renal capsule (34). It is important to note, however, that these effects occur at considerably higher doses than that used in this study. Furthermore, we have previously shown that treatment with anti-ICOS mAb in the absence of CsA does not improve islet allograft acceptance (35), suggesting that CsA is not detrimental to islet cell survival at the doses used in these studies.

Furthermore, the abundance of donor endothelial cells in the vascularized cardiac graft, as compared to very few numbers in the cellular islet graft may also influence the susceptibility of islets to rejection. The potential importance of endothelial cells in facilitating cardiac allograft survival is based on two important observations. First, endothelial cells have been shown express Fas ligand, which may play a critical role in protecting vascularized allografts from rejection by alloreactive T cells (36). Endothelial cells are the first allogeneic cells to interact with recipient lymphocytes, and binding of Fas ligand on endothelial cells to Fas on circulating allospecific T cells may trigger the T cell to undergo apoptosis. Therefore, through the regulation of T cell death, endothelial cells may render vascularized allografts less susceptible to rejection. The second observation is that endothelial cells have been shown to constitutively express high levels of the ligand for ICOS (37), suggesting that perhaps the ICOS costimulatory pathway may play an important role in endothelium-mediated T cell activation. Therefore, the differential expression of this ligand between endothelial cells and islet cells may influence the effectiveness of ICOS costimulation blockade-based strategies. However, our data suggest that the more important factor determining graft outcome appears to be the site of transplantation.

Finally, it has been shown that dramatically fewer, approximately 6000-fold less, alloreactive T cells are required to reject islet allografts compared to cardiac allografts (11). This important observation suggests that any strategy to induce tolerance through the reduction of allospecific clone size may be less effective in islet than cardiac allografts, depending on the extent of allospecific T cell deletion. Therefore, perhaps the blockade of ICOS costimulation in combination with CsA in our model may sufficiently reduce the alloreactive clone size to the threshold below the quantity required for cardiac allograft rejection, but not for islet allograft rejection. The challenge of sufficiently reducing the allospecific clone size to allow for renal subcapsular islet allograft acceptance is compounded by the presence of intact lymphatic drainage in the recipient kidney. By providing the required environment for T cell activation and differentiation, secondary lymphoid organs pose an additional barrier to achieving tolerance through costimulation blockade-based strategies. This was confirmed by demonstrating that splenectomy and resection of the draining lymph node to the kidney can significantly improve islet allograft survival under anti-ICOS mAb and cyclosporine therapy, with 30% of recipients achieving permanent graft acceptance. Presumably, not all allografts are accepted long-term due to the presence of other sites of intact lymphatic drainage such as the para-aortic lymph nodes. Nevertheless, elimination of some degree of secondary lymphoid tissue may reduce the capacity for effective alloreactive T cell activation to a level below that required to mediate rejection. In summary, the findings in this study elucidate the importance of graft site, with specific implication to the presence of secondary lymphoid organs, in the susceptibility of renal subcapsular islet allografts to rejection. Moreover, the dependence on secondary lymphoid tissue appears to be increased under conditions of costimulation blockade, suggesting that the presence of intact lymphatic drainage render islet allografts more resistant to costimulation blockade-based strategies for tolerance induction.

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CHAPTER FIVE

MULTIPLE COMBINATION THERAPIES INVOLVING BLOCKADE OF ICOS/B7RP-1 COSTIMULATION FACILITATE LONG-TERM ISLET ALLOGRAFT SURVIVAL

Sulaiman A. Nanji MD[1], Wayne W. Hancock MD, PhD[2], Colin C. Anderson PhD[1],
Andrew B. Adams PhD[3], Bin Luo MD[1], Colleen D. Schur BSc[1],
Rena L. Pawlick BSc[1], Liqing Wang MD, PhD[2], Anthony J. Coyle PhD[4],
Christian P. Larsen MD, PhD[3], A.M. James Shapiro MD, PhD[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada
[2] Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and University of Pennsylvania School of Medicine, Philadelphia, PA, USA
[3] Department of Surgery, Emory University, Atlanta, GA, USA
[4] Millennium Pharmaceuticals, Inc., Cambridge, MA, USA

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5.1 INTRODUCTION

Progress in clinical islet transplantation with the "Edmonton Protocol" and other recent refinements have created enthusiasm for this approach as an effective therapy for selected patients with Type I diabetes (1). While the risk of malignancy and life-threatening infection has been very low thus far, complications including severe mouth ulceration, hypercholesterolemia, renal dysfunction and hypertension presently preclude more broad application in the earliest stages of diabetes, including transplantation in children. Therefore, strategies that favor minimal toxicity ultimately towards development of tolerance protocols remain a primary focus as islet transplantation evolves.

One promising strategy to prevent graft rejection and facilitate tolerance induction is the blockade of costimulatory signals to inhibit T cell activation. T cells play a pivotal role in orchestrating the allo-immune response to transplanted tissue and are critically dependent upon two distinct but synergistic signals for their activation and clonal expansion. The first signal involves antigen-specific signaling through the T cell receptor (TCR) upon engagement with appropriately presented antigen. The second signal, referred to as costimulation, is delivered through costimulatory molecules on the surface of T cells upon interaction with their ligands on the antigen presenting cells (APC). If the T cell recognizes antigen through its TCR without adequate costimulation, it can become anergic and undergo apoptosis (2-4).

The CD28:B7 and CD40:CD40L costimulatory pathways play crucial roles in regulating T cell immune responses. CD28 is expressed constitutively on T cells and ligation with either B7-1 (CD80) or B7-2 (CD86) on the APC results in delivery of signals that promote clonal expansion and effector function of T cells (2). CD40:CD40L interactions serve to indirectly enhance costimulation via CD28 by upregulating the expression of B7 molecules, and also induce expression of adhesion molecules and inflammatory cytokines that participate in T cell activation (5). Blockade of CD28 or CD40L signaling with CTLA4-Ig or CD40L mAb, respectively, facilitates long-term allograft survival in a variety of transplantation models. Furthermore, the immunosuppressive agent rapamycin, a potent inhibitor of T cell proliferation (6), has been shown to synergize with the blockade of these costimulatory signals in tolerance induction (7).

In recent years, a structurally related molecule to CD28 has been identified and termed inducible costimulator (ICOS) (8). Despite its structural similarity with CD28, ICOS does not interact with the ligands B7-1 and B7-2, but with a novel molecule in the B7 family, namely B7-related protein-1 (B7RP-1) (9, 10). B7RP-1 is expressed on B cells, macrophages and dendritic cells, and ligation with ICOS results in the delivery of signals that promote T cell proliferation and differentiation (9, 11, 12). Unlike CD28, ICOS is not expressed constitutively on naïve T cells but is induced on T lymphocytes upon activation, suggesting that it is important in regulating activated T cells (13). While the exact role of ICOS in regulating immune responses remains to be further elucidated, its discovery has revealed a new target for manipulating T cell activation. To date, very few studies investigating the role of ICOS in transplant models have been reported, and although the blockade of this molecule has been shown to be effective in prolonging heart (14) and liver (15) allograft survival, much has still to be learned regarding the underlying mechanisms of action of this new pathway in vivo.

In a fully MHC-mismatched mouse model of islet transplantation, we demonstrated that while monotherapy with CTLA4-Ig, CD40L mAb or rapamycin enhanced islet allograft survival, the addition of a blocking anti-ICOS mAb to these single agent therapies resulted in significantly improved islet allograft survival. Mechanistic studies and donor re-challenge were performed in mice treated with anti-ICOS mAb and rapamycin therapy and revealed a dramatic inhibitory effect on the initial expansion and function of allo-reactive T cells, without the induction of donor-specific immunological tolerance. Rather, this combination strategy induces a state of operational tolerance where islet allografts are accepted long-term (>100 days) without the need for maintenance immunosuppressive therapy.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

Adult C57BL/6 (H-2^b), BALB/c (H-2^d), and CBA/JCr (H-2^k) male mice were obtained from Charles River Canada. Mice were housed under standard conditions and cared for in accordance with the guidelines established by the Canadian Council on Animal Care.

5.2.2 INDUCTION OF DIABETES AND ISLET TRANSPLANTATION

C57BL/6 recipient mice were rendered chemically diabetic by a single intravenous injection of streptozotocin (200 mg/kg, Sigma-Aldrich, Canada). Fully MHC-mismatched donor BALB/c islets were isolated by collagenase digestion (1 mg/ml, Sigma-Aldrich) followed by Ficoll purification (16, 17) (Sigma-Aldrich). Approximately 500 islets were then transplanted under the left renal capsule of diabetic recipient mice. Allograft function was monitored by serial blood glucose measurements. Successful engraftment was defined by correction of serum glucose level to <8 mmol/L by the third day post-transplant, and graft rejection was defined as a rise in serum glucose >15 mmol/L for two consecutive days.

5.2.3 REAGENTS AND TREATMENT PROTOCOLS

CD40L mAb (CD154, clone MR-1) was purchased from BioExpress (West Lebanon, NH) and production and characterization of the non-depleting anti-ICOS mAb (12A8) has been described previously (14). Cyclosporine (CsA) was purchased from the University of Alberta Pharmacy, CTLA4-Ig was donated by Bristol-Myers Squibb and rapamycin was provided by Wyeth Canada. To evaluate the role of these agents in islet allograft rejection, recipient mice were treated either with CD40L mAb (0.25 mg, days 0, 2, 4 and 6), CTLA4-Ig (0.25 mg, days 0, 2, 4, 6), CsA (10mg/kg/d x 14 days), or rapamycin (0.2 mg/kg/d x 14 days) as single agents. Comparable groups of mice were treated with the addition of anti-ICOS mAb (0.1 mg/d x 14 days) to each of the single agent therapies. All reagents were administered i.p. beginning on the day of transplantation.

5.2.4 MIXED LYMPHOCYTE REACTIONS (MLRS)

Splenocytes were cultured in duplicate wells containing 2×10^5 responder cells with various dilutions of irradiated (1500 Rad) stimulator cells. Responder cells were obtained from naïve and tolerant mice, while stimulator cells were derived from C57BL/6 (syngeneic), BALB/c (donor) and CBA/J (3rd party) mice. After culture at 37 °C for 3 days, cells were pulsed for 18 h with 1 μCi ^{3}H thymidine/well, harvested and thymidine incorporation determined. To assess the presence of regulatory T cells in tolerant mice, splenocytes from tolerant and naïve mice were co-cultured in MLRs to donor antigen. The ratio of tolerant to naïve cells in co-culture reactions was increased to ensure that adequate regulatory cell numbers were present to detect a potential suppressive effect. Additional MLRs with only naïve and tolerant cells were included to control for changing total cell number and to assess the proliferation of each cell population individually.

5.2.5 *CYTOTOXIC LYMPHOCYTE REACTIONS (CTLS)*

Cytotoxic responses were assayed by the JAM Test as previously described (18). Briefly, 5×10^6 spleen cells from C57BL/6 naïve and tolerant mice (responders) were stimulated for 5 days with 2×10^6 irradiated BALB/c (donor) spleen cells. Con A blast targets were set up 40 hours prior to the CTL assay by culturing 1.5×10^6 naïve syngeneic and BALB/c spleen cells with Con A (1.25 μg/ml), then labeling with ^{3}H-thymidine. Lysis of target cells was tested at various responder to target ratios.

5.2.6 *CONFIRMATION OF GRAFT FUNCTION AND RE-TRANSPLANTATION*

Long-term graft function of normoglycemic mice after 100 days was confirmed by a return to hyperglycemia following nephrectomy of the kidney bearing the islet graft. To test for immunological tolerance, nephrectomized mice underwent re-transplantation of same donor-strain islets into the remaining contralateral kidney. No immunosuppressive therapy was given and blood glucose was serially monitored to detect for graft rejection.

5.2.7 *IMMUNOPATHOLOGY*

Following nephrectomy of graft-bearing kidneys, one half of the islet allograft was embedded in Histo Prep (Fisher Scientific, Canada) and frozen in liquid nitrogen for storage at –70 °C until sectioning. The other half of the graft was stored in formalin and then paraffin embedded. Cryostat sections were analyzed by immunohistology for the presence of ICOS expression by graft infiltrating leukocytes (14) and paraffin sections were stained with hematoxylin and eosin to assess overall cellularity.

5.2.8 CFSE LABELING AND IN VIVO QUANTIFICATION OF T CELL PROLIFERATION

Spleens and mesenteric lymph nodes were harvested from naïve C57BL/6 mice and prepared as a single cell suspension. After RBC lysis and nylon wool passage for T cell purification, cells were re-suspended and incubated with 10 μM of the tracking fluorochrome CFSE (Molecular Probes, Eugene, OR). Approximately 20 x 10^6 CFSE-labeled cells were then adoptively transferred to lethally irradiated (1800 Rad) BALB/c recipients. Mice were subsequently allocated into four separate treatment groups: 1) no treatment; 2) anti-ICOS mAb alone (0.1 mg/day); 3) rapamycin alone (0.2 mg/kg/day); and 4) anti-ICOS mAb (0.1 mg/day) + rapamycin (0.2 mg/kg/day). A fifth group consisting of syngeneic C57BL/6 control mice also received CFSE-labeled cells. Approximately 72 hours after cell transfer, mice were sacrificed and their splenocytes were harvested and prepared as single cell suspensions. The RBCs were lysed and the remaining cells were stained with either anti-CD4 (PharMingen, San Diego CA) or anti-CD8 (Caltag, Burlingame CA) biotinylated antibody. Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (Becton-Dickinson, Braintree, MA). By gating onto $CD4^+$ $CFSE^+$ cells and $CD8^+$ $CFSE^+$ cells, the proliferation of $CD4^+$ and $CD8^+$ T cells in each separate generation of dividing cells could be determined according to their CFSE profiles.

5.2.9 ASSESSMENT OF ALLOREACTIVE T CELL PROLIFERATION

The frequency of T cells that went through 4 or more (maximum 8) divisions was calculated as previously described (19). In brief, peaks were labeled according to the number of times the cells had divided (*n*). A T cell that divides *n* times generates 2^n daughter cells. Therefore, to obtain the number of precursors for each individual peak, the total number of daughter cells in each peak is divided by 2^n. For each mouse receiving allogeneic cells, the original number of precursors that divided and gave rise to the daughter cells in each peak was determined. The number of precursors for peaks 4–8 were added together and divided by the total number of initial precursors to generate a precursor frequency of cells that undergo 4 or more divisions. The divisions 4 to 8 were chosen based on the observation that syngeneic transferred cells do not proliferate detectably beyond three divisions.

5.2.10 INTRACELLULAR STAINING OF IFN-γ PRODUCTION AS A MEASURE OF ALLOSPECIFIC T CELL RESPONSES

Naive C57BL/6 mice received full-thickness BALB/c skin grafts, and were subsequently allocated into four separate treatment groups: 1) no treatment; 2) anti-ICOS mAb alone (0.1 mg/day); 3) rapamycin alone (0.2 mg/kg/day); and 4) anti-ICOS mAb (0.1 mg/day) + rapamycin (0.2 mg/kg/day). Ten days after transplantation and treatment, mice were sacrificed and their splenocytes were harvested and prepared as single cell suspensions for evaluation of allospecific T cell responses. This was accomplished using an ex-vivo re-stimulation technique using BALB/c splenocytes as stimulators at a 1:1 (stimulator:responder) ratio. Cells were then stained with anti-IFN-γ and either anti-CD4 or anti-CD8 using the Cytofix/Cytoperm kit according to the manufacturer's instructions (Pharmingen, San Diego CA). Flow cytometry was performed on a FACSCalibur and data was analyzed using CellQuest software.

5.2.11 STATISTICAL ANALYSIS

Graft survival was analyzed using the Kaplan-Meier method, and statistical comparisons among groups were performed using the log rank test. Comparisons of precursor frequencies and of IFN-γ production among different groups were done using the non-parametric Mann-Whitney *U* test (SPSS version 10.0, Chicago, IL).

5.3 RESULTS

5.3.1 NEITHER COSTIMULATION BLOCKADE NOR IMMUNOSUPPRESSIVE THERAPY INDUCES INDEFINITE ISLET ALLOGRAFT SURVIVAL

We first investigated the effect of CD40L mAb, CTLA4-Ig, rapamycin or CsA treatment as single agent therapies on islet allograft rejection. Fully MHC-mismatched C57BL/6 mice were rendered chemically diabetic and transplanted with approximately 500 BALB/c islets under the renal capsule. Compared to untreated controls, mice treated with anti-CD40L, CTLA4-Ig or rapamycin demonstrated significantly prolonged islet allograft survival ($p<0.05$; Figure 5-1), whereas CsA had no significant effect on graft survival. However, with the exception of CD40L mAb, these agents were only marginally effective at conferring indefinite graft acceptance (>100 d) when used as individual therapies, indicating that blockade of a single pathway is ineffective at completely preventing islet graft rejection.

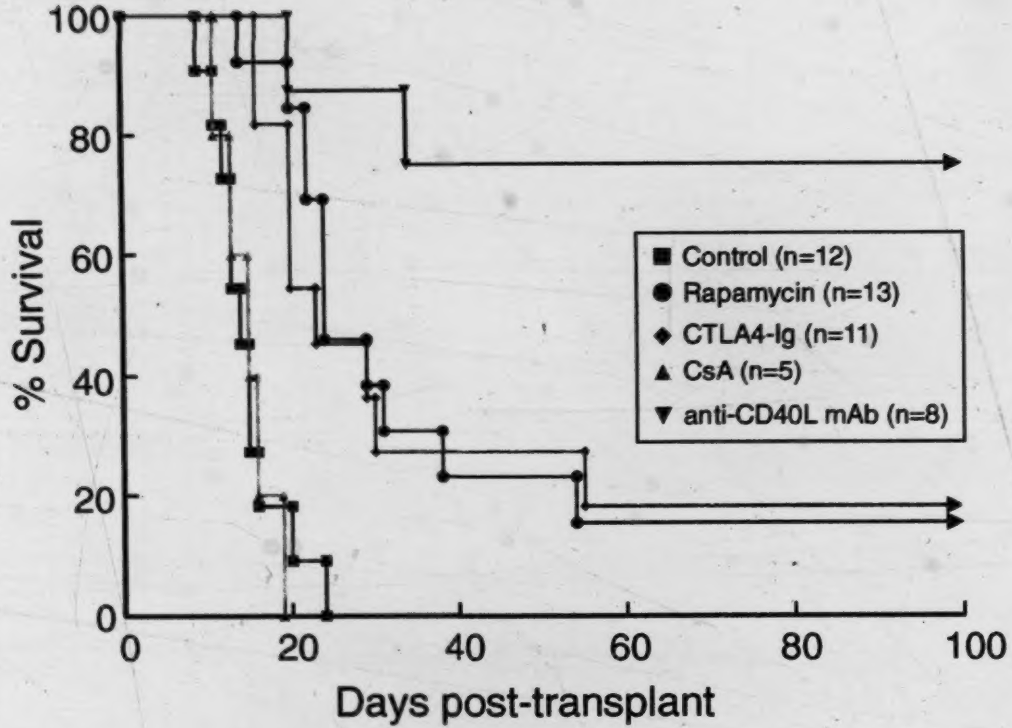


Figure 5-1: Islet allograft survival.
500 BALB/c islets were transplanted under the renal capsule of streptozotocin treated diabetic C57BL/6 mice. Monotherapy with anti-CD40L (MST=82), CTLA4-Ig (MST=23), or rapamycin (MST=24) led to significantly prolonged graft survival compared to untreated control mice (MST=14, $p<0.05$), while CsA had no significant effect on graft survival (MST=15).

5.3.2 *ANTI-ICOS MAB IN COMBINATION WITH CD40L MAB, CTLA4-IG OR RAPAMYCIN FACILITATES INDEFINITE ISLET ALLOGRAFT SURVIVAL*

The recent discovery of the ICOS molecule has added new options in efforts to use costimulation blockade to facilitate allograft survival. To explore the potential relevance of this pathway in islet allograft rejection, we undertook immunohistologic analyses for ICOS upregulation in rejected islet allografts of mice treated with CTLA4-Ig or CD40L mAb costimulation blockade, or rapamycin or CsA immunosuppressive therapy. We found that ICOS expression was strikingly increased on host mononuclear cells infiltrating islet allografts in both untreated and treated recipients (Figure 5-2), suggesting that ICOS signaling may provide an alternate escape pathway in mediating islet allograft rejection in mice treated with either costimulation blockade or immunosuppressive therapy. To test this theory, we studied the effect of combining the

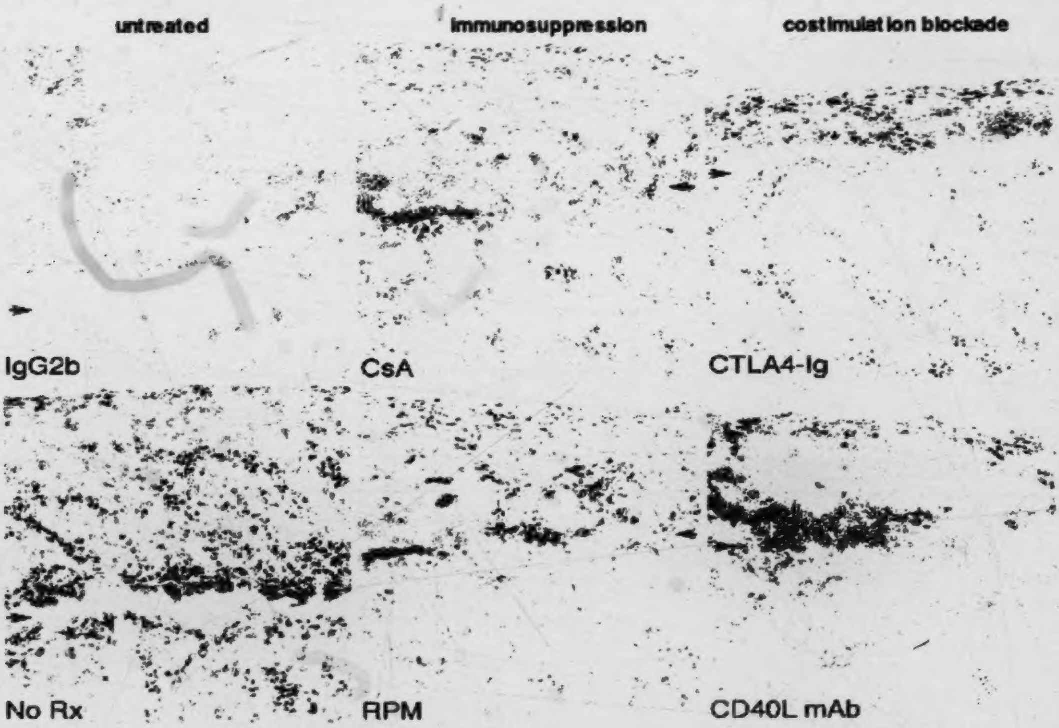


Figure 5-2: ICOS expression by host MNC during islet allograft rejection.
In contrast to the lack of staining using an isotype control IgG2b mAb, islet allografts in untreated (No Rx) mice, or islet allograft recipients treated with CsA, rapamycin (RPM), CTLA4-Ig or CD40L mAb showed dense expression of ICOS by infiltrating host MNC at the time of rejection. Arrows in each case indicate the border between underlying renal cortex and the islets placed below the renal capsule. (Cryostat sections, hematoxylin counterstain, x200 original magnifications, representative of at least 3 grafts/group).

single agent therapies with an anti-ICOS mAb. As depicted in Figure 5-3, transplanted mice treated with the combination of anti-ICOS mAb demonstrated a significant improvement in graft survival ($p<0.05$), indicating that the ICOS-B7RP-1 pathway indeed plays an important role in the rejection of islet allografts. Use of anti-ICOS mAb did not potentiate the effects of CsA in this model, at least at the sub-therapeutic dosage of CsA tested. Long-term graft function was confirmed in mice maintaining euglycemia beyond 100 days as nephrectomy of the graft-bearing kidney resulted in a return to hyperglycemia in all cases (n=6).

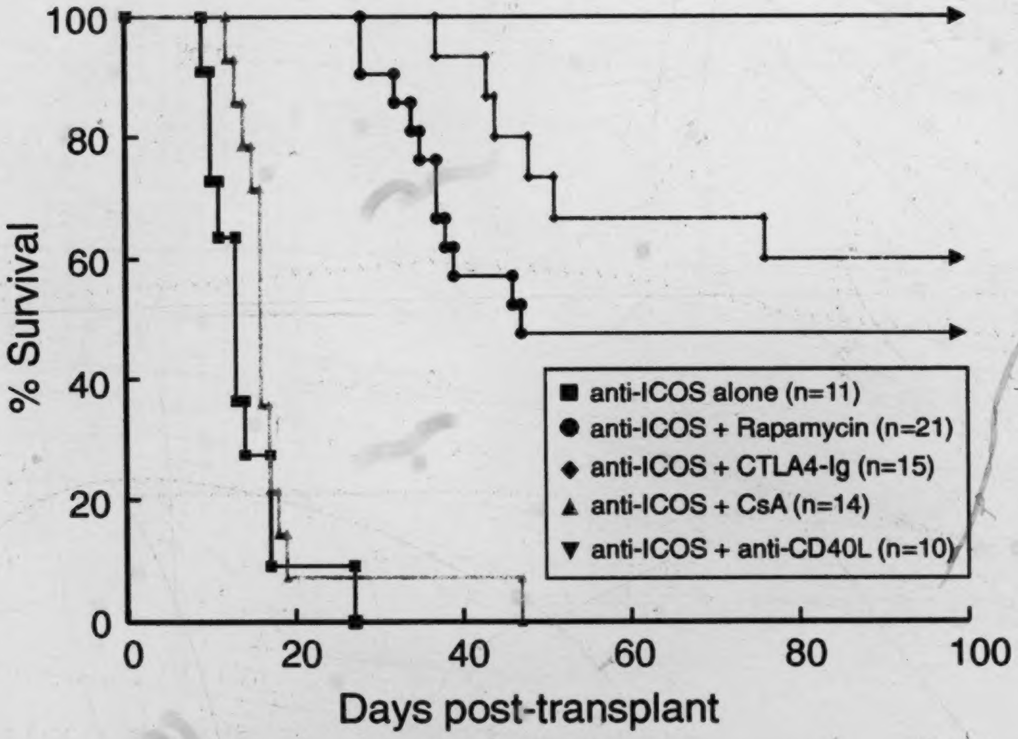


Figure 5-3: Combination of anti-ICOS mAb with monotherapies significantly enhances islet allograft survival.
Anti-ICOS with CTLA4-Ig (MST=80) or rapamycin (MST=47) resulted in significantly improved graft survival compared to CTLA4-Ig (MST=23), rapamycin (MST=24), or anti-ICOS (MST=13) alone ($p<0.05$). The addition of anti-ICOS mAb to CD40L mAb led to indefinite survival in all treated mice, whereas anti-ICOS mAb did not potentiate the effects of CsA (MST=16).

5.3.3 ANTI-ICOS MAB AND RAPAMYCIN THERAPY DOES NOT INDUCE DONOR-SPECIFIC UNRESPONSIVENESS

Although anti-ICOS mAb effectively enhanced graft survival in virtually all combination treatment groups, we focused on immunological mechanisms in mice treated with anti-ICOS mAb and rapamycin as this strategy may have the greatest potential for clinical translation. We first sought to evaluate the robustness of the tolerant state induced by anti-ICOS mAb and rapamycin. T cell responses of long-term engrafted mice were analyzed in comparison to naïve responses by mixed lymphocyte reactions to both donor (BALB/c) and third party (CBA/J) antigen (Figure 5-4A). In this assay, lymphocytes from long-term engrafted mice had at most only a slight reduction in the proliferative response to donor and third party antigen as compared to the response from naïve mice. This suggests that islet allograft survival was not a consequence of a long-term donor-specific unresponsiveness. Moreover, in vitro CTL assays were completed to evaluate anti-donor T cell cytotoxic responses in mice demonstrating indefinite graft acceptance. In this assay, the ability of naïve and long-term engrafted mice to mount CTL responses was essentially equivalent (Figure 5-4B).

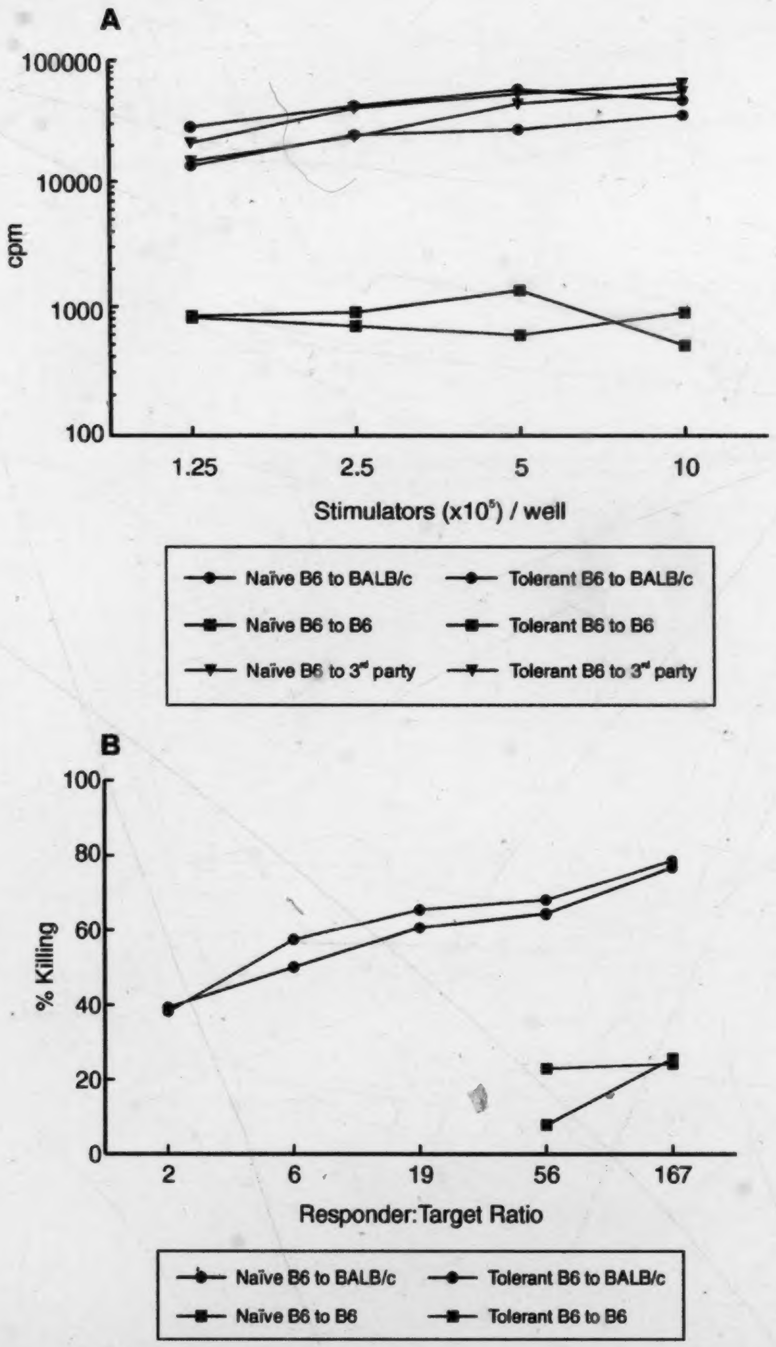


Figure 5-4: In vitro evaluation of T cell responses in long-term engrafted mice treated with anti-ICOS mAb and rapamycin.
Lymphocytes from long-term engrafted mice demonstrate robust proliferative (A) and cytotoxic (B) responses comparable to naïve responses.

The maintenance of normal allograft function in light of these in vitro findings could possibly be attributed to regulatory T cells. These cells have been described in several models of transplantation tolerance (20-22), including costimulation blockade based strategies (23, 24). In this study, an MLR assay was used to test for the presence of regulatory T cells in mice treated with anti-ICOS mAb and rapamycin. Splenocytes from long-term engrafted mice were co-cultured with splenocytes from naïve mice and proliferation was evaluated in response to donor antigen. As demonstrated in Figure 5-5, the ability of naïve T cells to proliferate in response to donor antigen either alone or in the presence of lymphocytes from long-term engrafted mice is essentially equivalent.

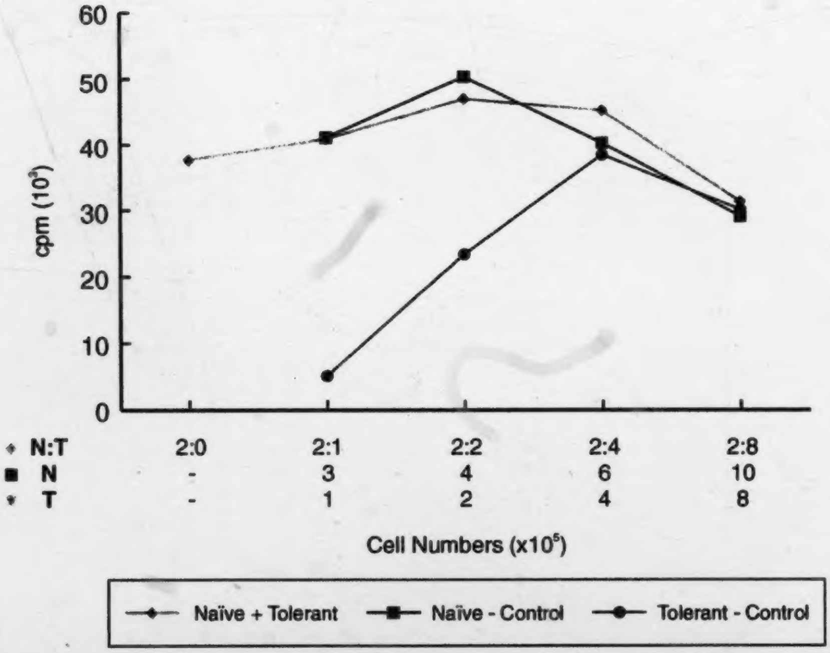


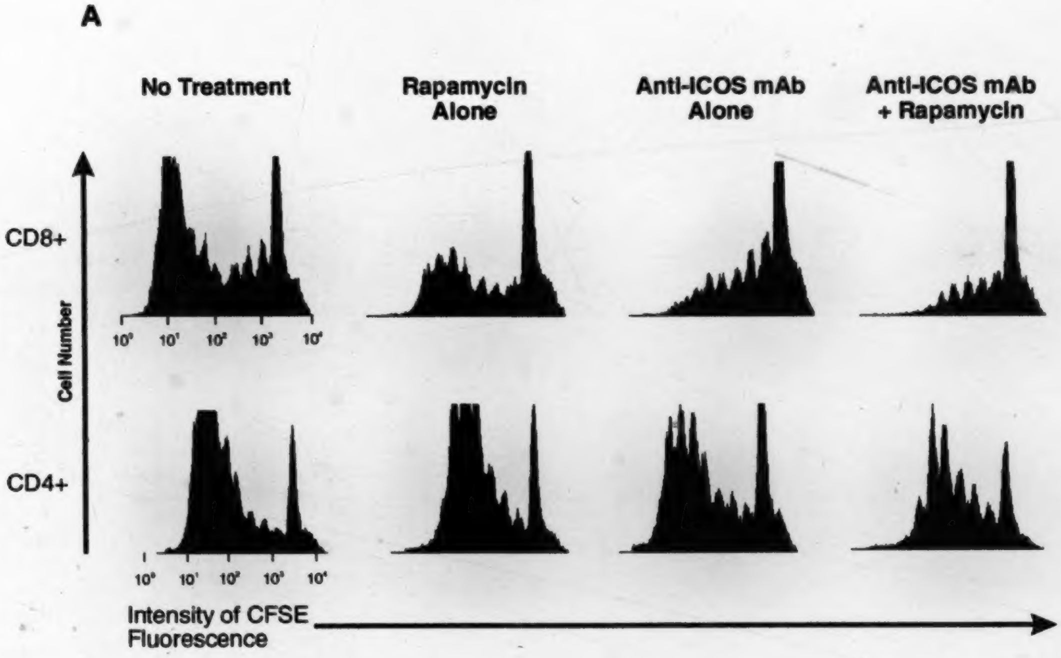
Figure 5-5: Long-term engrafted mice treated with anti-ICOS and rapamycin do not demonstrate in vitro evidence of regulatory T cell activity.
A coculture MLR assay was performed to test for the presence of regulatory T cells. As shown on the x-axis, increasing numbers of splenocytes from tolerant mice were mixed with a fixed number of splenocytes from naïve mice (N:T), and proliferation was evaluated in response to donor alloantigen. MLRs with only naïve (N) and tolerant (T) cells were included to control for changing total cell number, and to assess the proliferation of each cell population individually.

Even with increasing numbers of "tolerant" lymphocytes in co-culture, suppression of the MLR in response to alloantigen was not detected. Furthermore, additional co-culture MLRs were performed where the total number of cells in each well was kept constant but the proportion of naïve:"tolerant" cells was serially increased. Even with this approach, there was no suppression of the MLR response to donor-antigen (data not shown). Therefore, based on the above assays, these results indicate that regulatory T cells could not be detected in the spleen of mice treated with anti-ICOS mAb and rapamycin.

Finally, having shown that treatment with anti-ICOS mAb and rapamycin did not eliminate anti-donor T cell responses beyond 100 days, recipients with prolonged graft function were then re-challenged. After graft nephrectomy and return of hyperglycemia, a second donor-specific islet graft was placed in the remaining contralateral kidney, without further immunosuppressive therapy (n=5). Four of the five re-transplanted grafts were rejected at a median of 30 days post-transplant (14, 21, 30, 31, >100 days), suggesting that immunological tolerance was not achieved in this model. Rather, a state of operational tolerance is established, where the recipient is capable of maintaining long-term graft function in the absence of maintenance immunosuppressive therapy.

5.3.4 TREATMENT WITH ANTI-ICOS MAB AND RAPAMYCIN BLOCKS CD4$^+$ AND CD8$^+$ T CELL ALLORESPONSES

Having established that combined treatment with anti-ICOS mAb and rapamycin does not induce long-term donor-specific hyporesponsiveness, we hypothesized that the treatment may act primarily on the early acute anti-donor responses. To test this, an in vivo allo-proliferation assay was set up to evaluate the impact of this treatment on the initial ability of allospecific T cells to replicate (25). As depicted in Figure 5-6A, CD4$^+$ and CD8$^+$ T cells in untreated mice demonstrated strong proliferative responses to BALB/c hosts with up to 8 discrete divisions. In contrast, cells transferred into syngeneic hosts underwent negligible proliferation, with no cells progressing beyond three divisions (Figure 5-6B). Histograms depicting cellular replication in treated mice revealed a dramatic reduction in the strength of both CD4$^+$ and CD8$^+$ T cell proliferation (Figure 5-6A). The most striking decay in response is illustrated in mice treated with combined anti-ICOS mAb and rapamycin, particularly in the CD8$^+$ subpopulation.



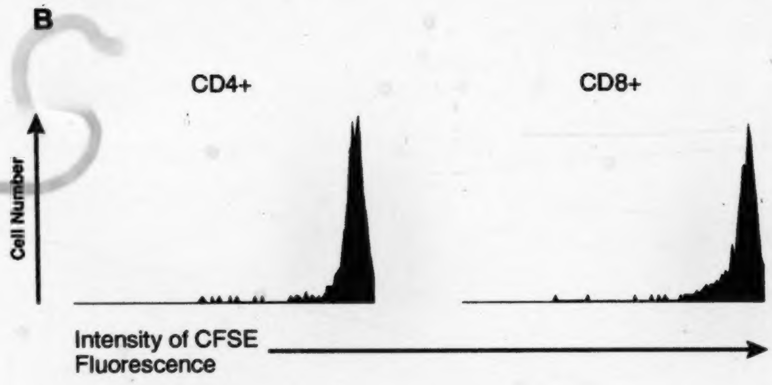


Figure 5-6: In vivo allo-proliferation assay under anti-ICOS and/or rapamycin treatment (CFSE)
Purified T cells from spleens and mesenteric nodes of naïve C57BL/6 mice were labeled with the fluorescent dye CFSE and adoptively transferred into supralethally irradiated BALB/c (A) or syngeneic B6 (B) mice. Mice were subsequently allocated into 5 treatment groups consisting of three mice each: 1) no treatment, 2) anti-ICOS mAb alone, 3) rapamycin alone, 4) anti-ICOS mAb + rapamycin, and 5) syngeneic control. Approximately 72 hours after allogeneic transfer, splenocytes were harvested and stained for expression of CD4 and CD8. By gating on CD4$^+$ CFSE$^+$ cells or CD8$^+$CFSE$^+$ cells, the proliferation of CD4$^+$ and CD8$^+$ T cells in each separate generation of dividing cells could be determined according to their CFSE profiles. In (A) and (B), the peak on the far right represents undivided cells, and consecutive peaks with decreasing fluorescence represent successive divisions.

Analysis of precursor frequencies of T cells undergoing 4 to 8 divisions for individual groups demonstrated a significant reduction in both CD4$^+$ and CD8$^+$ allo-specific T cell proliferation in mice treated with anti-ICOS mAb and/or rapamycin (Figure 5-7). Compared to untreated recipients, mice treated with rapamycin alone had a 28% and 43% reduction in allo-reactive CD4$^+$ and CD8$^+$ T cell proliferation, respectively ($p<0.05$). Mice treated with anti-ICOS mAb alone demonstrated an even greater reduction, 60% and 82% reduction in CD4$^+$ and CD8$^+$ T cell proliferation, respectively ($p<0.05$). Combined therapy with anti-ICOS mAb and rapamycin did not appear to influence CD4$^+$ T cell proliferation more than anti-ICOS mAb alone, as evidenced by the comparable precursor frequency. However, with respect to the CD8$^+$ sub-population, there is an even further reduction in precursor frequency using combination therapy (91% reduction, $p<0.05$). The dramatic reduction in alloreactive CD4$^+$ and CD8$^+$ T cell proliferation with ICOS blockade alone has also been recently demonstrated in a murine model of cardiac allo-transplantation, however, it was not demonstrated whether the anti-ICOS mAb used was depleting or not (26).

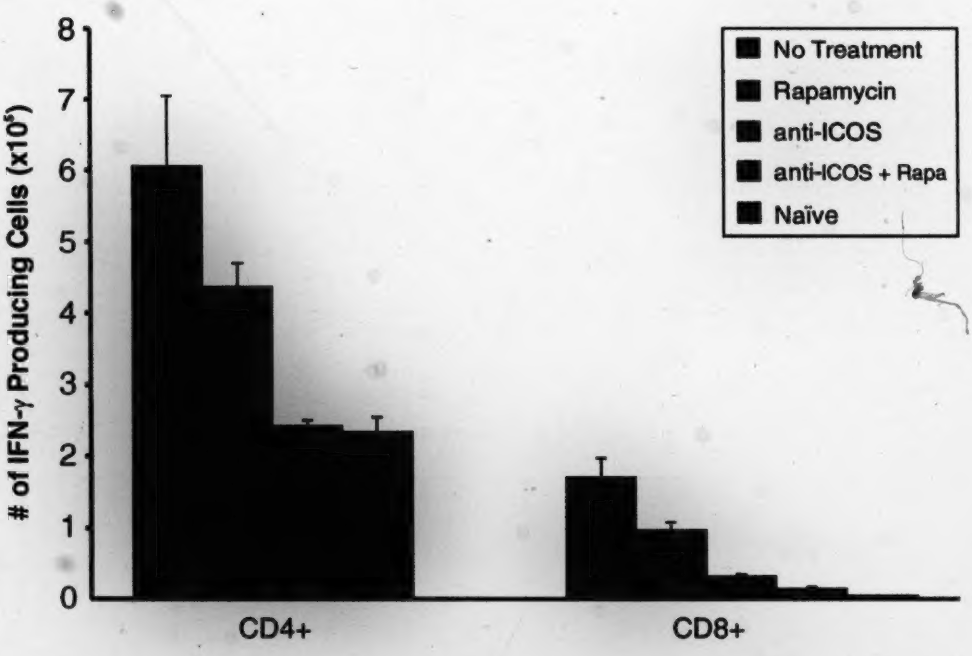


Figure 5-7: Treatment with anti-ICOS mAb and/or rapamycin significantly reduces proliferation frequencies of both CD4$^+$ and CD8$^+$ T cells.
Calculation of precursor frequency of T cells undergoing 4 or more (maximum visible 8) divisions reveals that combined treatment with anti-ICOS mAb and rapamycin has the most potent inhibitory effect on CD4$^+$ and CD8$^+$ allo-specific T cell proliferation.

5.3.5 MICE TREATED WITH ANTI-ICOS MAB AND RAPAMYCIN DEMONSTRATE A REDUCED FREQUENCY OF IFN-γ PRODUCING T CELLS

While there was a profound inhibitory effect of combined treatment on the early donor-specific proliferative response, it was possible that differentiation to effector function remained unaffected. To test this, we examined the effect of anti-ICOS mAb and/or rapamycin therapy on the ability of mice to generate allospecific IFN-γ producing T cells during induction therapy. Analysis of flow cytometry data from treated mice revealed a reduction in the frequency of T cells capable of producing IFN-γ in response to alloantigen. When drugs were administered as single agents, rapamycin resulted in a greater reduction in the number of IFN-γ producing $CD4^+$ and $CD8^+$ T cells than did anti-ICOS mAb treatment. However, the combination of these agents resulted in the most potent decrease in the generation of IFN-γ producing T cells (89% and 90% reduction in the frequency of $CD4^+$ and $CD8^+$ IFN-γ producing T cells, respectively; $p<0.05$; Figure 5-8A and B).

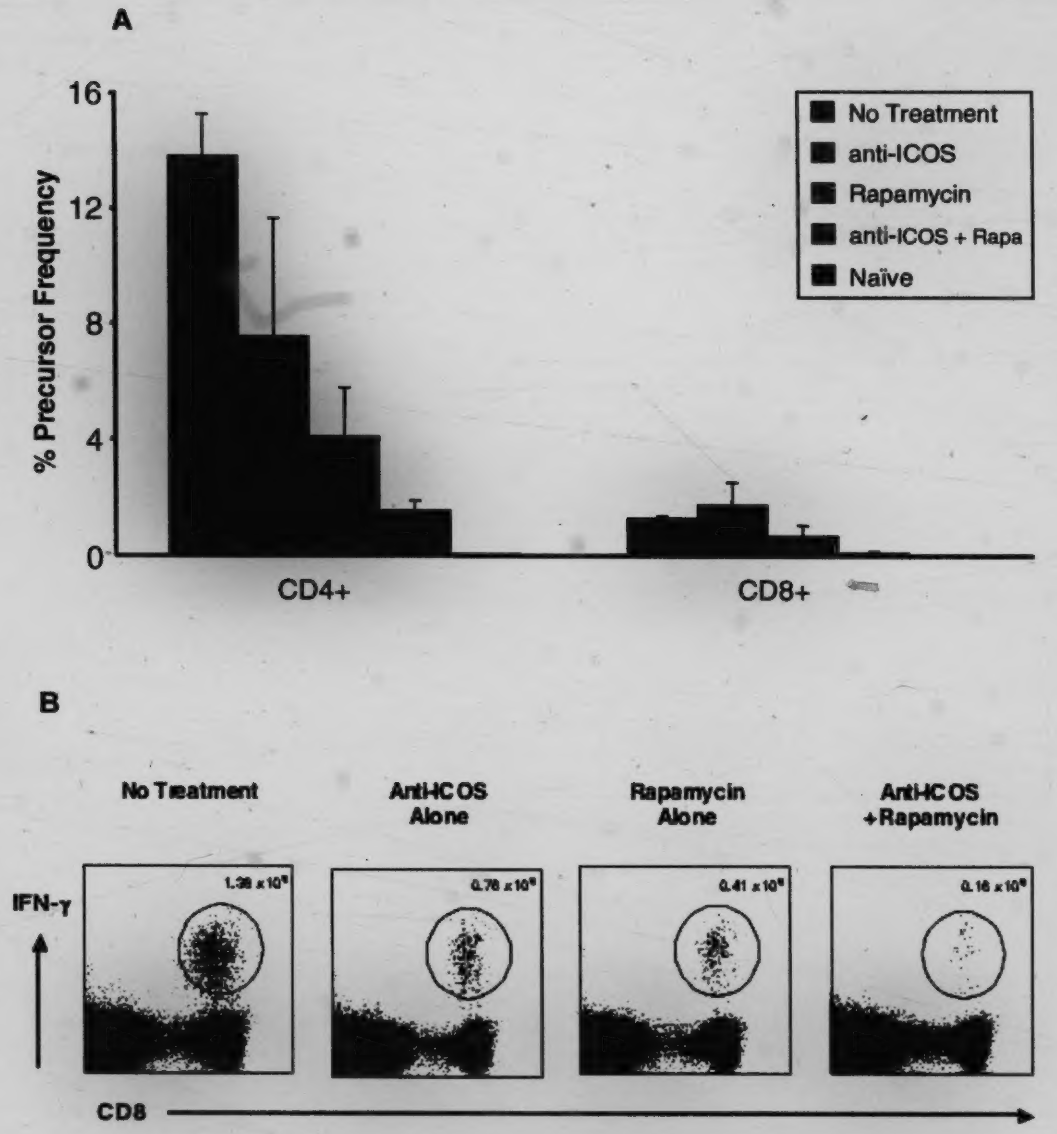


Figure 5-8: Treatment with anti-ICOS mAb and/or rapamycin significantly reduces the frequency of allospecific IFN-γ producing CD4$^+$ and CD8$^+$ T cells.
(A) Combined treatment with anti-ICOS mAb and rapamycin has the greatest reduction in the number of IFN-γ producing CD4$^+$ and CD8$^+$ T cells. Naive C57BL/6 mice received full-thickness BALB/c skin grafts and were subsequently allocated into four separate treatment groups consisting of at least 2 mice each: 1) no treatment; 2) anti-ICOS mAb alone; 3) rapamycin alone; and 4) anti-ICOS mAb + rapamycin. Ten days after transplantation and treatment, the frequency of IFN-γ producing CD4$^+$ and CD8$^+$ T cells was evaluated in response to donor antigen by intracellular cytokine staining and subsequent analysis by flow cytometry. (B) Example of flow cytometry data from mice in four separate treatment groups, depicting the frequency of CD8$^+$ T cells capable of generating IFN-γ in response to donor-antigen. The number of CD8$^+$ IFN-γ$^+$ cells in each gate is included in the upper right corner of each plot.

5.4 DISCUSSION

The ongoing identification of new costimulatory molecules suggests that the immune system has an inherent functional redundancy to ensure that adequate T cell activation occurs in the presence of foreign antigen. The corollary is that more effective blockade of T cell activation and tolerance induction may require combined blockade of two or even more costimulatory signals. This concept has been validated in previous small and large animal studies where combined blockade of CD28 and CD40L signaling (27, 28) or CD40L and ICOS signaling (14) resulted in improved graft survival. In our murine model of islet transplantation, we have demonstrated that while treatment with rapamycin or the blockade of CD28 or CD40L signaling can enhance islet allograft survival, rejection of the allograft cannot be completely prevented using single agent therapies. Immunohistologic analysis of rejected grafts revealed increased expression of ICOS by host MNC, suggesting that this costimulatory molecule may act to provide an alternate pathway for T cell activation leading to graft rejection. The combination of an anti-ICOS blocking mAb with the single agent therapies initially tested resulted in significantly improved islet allograft survival, confirming that ICOS signaling does indeed play a role in islet allograft rejection. Given the importance of ICOS signaling in antibody class switching and germinal center formation (29), a possible contributory mechanism in facilitating graft survival in this study may be the inhibition of alloantibody formation.

Although anti-ICOS mAb led to improved graft survival when used in combination with either CTLA4-Ig, anti-CD40L or rapamycin, we chose to explore immunological mechanisms in mice treated with combined anti-ICOS mAb and rapamycin. The rationale for further investigation in this combination was based on three previously described findings. Firstly, it has been shown that rapamycin synergizes with costimulation blockade to prolong allograft survival through enhanced apoptosis of alloreactive T cells (7). Secondly, ICOS signaling may act synergistically with IL-2 mediated signal transduction in T cell activation (30), strengthening the rationale for blockade of both pathways. Thirdly, this combination was chosen based on rapamycin's proven efficacy clinically in islet transplant patients (1).

Evaluation of the tolerant state in mice treated with anti-ICOS mAb and rapamycin demonstrated that mice with prolonged graft survival were capable of rejecting second same donor re-transplants, indicating that immunological tolerance was not achieved. In this model, mice receiving a second graft first underwent nephrectomy of the primary graft, thereby creating a window of time where donor antigen was absent in the recipient prior to re-challenge. In models dependent on clonal anergy of T cells for the maintenance of tolerance, it has been suggested that the continual presence of antigen is required to sustain T cell anergy in vivo, and that the nonresponsive state can be reversed when the antigen is absent (31). Therefore, the blockade of ICOS costimulation in our model may have promoted a state of clonal anergy, and that the absence of alloantigen may have allowed a reversal of the nonresponsive state with subsequent rejection of second islet allografts. However, the notion that ICOS facilitates clonal anergy in our model seems unlikely since we found that operationally tolerant mice treated with anti-ICOS mAb and rapamycin demonstrated intact anti-donor proliferative and cytotoxic responses in vitro. This suggests that mechanisms other than anergy are responsible for the maintenance of peripheral tolerance in our model. It has been shown that the combination of rapamycin and costimulation blockade results in extensive apoptosis of proliferating T cells (7). Therefore, the survival benefit observed with anti-ICOS mAb and rapamycin therapy may be attributed in part to the peripheral deletion of donor-specific T cells. This rationale may also serve to explain why we observed a significant reduction in both donor-specific proliferation of T cells and the generation of IFN-γ producing T cells during induction therapy with anti-ICOS and rapamycin.

The discrepancy between proliferative and cytotoxic responses being profoundly inhibited during induction therapy with anti-ICOS mAb and rapamycin, while they appear to be intact in treated mice with long-term allograft survival may be attributed to the generation of new allo-reactive thymic emigrants. The maintenance of an operationally tolerant state despite the generation of new allo-specific T cells may be attributed to the lack of danger signals in the presence of a well-established graft (32). Another possible explanation for the maintenance of normal graft function in the absence of immunosuppression may be attributed to regulatory T cells. Although co-culture lymphocyte reactions failed to demonstrate the presence of regulatory T cells in the spleens of mice treated with anti-ICOS mAb and rapamycin, these findings do not exclude the possibility of an active regulatory T cell population functioning at the level of the graft (33). Immunopathology of cellular infiltrates in long-term accepted grafts revealed a peri-islet infiltrate of $CD4^+$ cells (data not shown), which might indicate the presence of a local regulatory subpopulation of cells exerting a protective suppressive effect on allo-aggressive T cells.

In summary, our findings suggest that combination therapy with blockade of the ICOS costimulation can enhance islet allograft survival, indicating that ICOS signaling plays an important role in islet allograft rejection.Further studies in mice treated with anti-ICOS mAb and rapamycin revealed that a state of operational, and not immunological tolerance is achieved, and that this combination has a potent inhibitory effect on acute allo-responses in vivo. While the exact mechanisms responsible for the maintenance of normal graft function in mice treated with anti-ICOS mAb and rapamycin have not been elucidated, early deletion of allo-reactive T cells due to costimulation blockade may allow for initial graft acceptance. This may be followed by progressive waning of costimulatory signals as a healthy graft becomes established, eliminating the need for continuous costimulation blockade. Further investigation into the importance of ICOS costimulation is clearly warranted given its potential therapeutic benefit in promoting successful islet transplantation. In view of the importance of overcoming both auto and allo-immunity barriers in islet transplantation, these studies should be actively pursued in the NOD mouse model, and such studies are currently underway in our laboratory.

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CHAPTER SIX

COMBINED BLOCKADE OF ICOS AND CD40L COSTIMULATION INDUCES DOMINANT TOLERANCE TO ISLET ALLOGRAFTS AND PREVENTS SPONTANEOUS AUTOIMMUNE DIABETES IN NOD MICE

Sulaiman A. Nanji MD[1], Wayne W. Hancock MD, PhD[2], Bin Luo MD[1],
Colleen D. Schur BSc[1], Rena L. Pawlick BSc[1], Lin Fu Zhu MD[1],
Colin C. Anderson PhD[1], A.M. James Shapiro MD, PhD[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada
[2] Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and University of Pennsylvania School of Medicine, Philadelphia, PA, USA

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6.1 INTRODUCTION

Complete T cell activation in response to antigenic stimulation requires two separate but synergistic signals. The first signal confers antigen specificity to the immune response and arises through the T cell receptor upon engagement with appropriately presented antigen. The second signal, known as costimulation, is delivered by one or more T cell surface receptors after interaction with their respective ligands on the antigen presenting cell (APC) (1, 2). Since a T cell that encounters antigen in the absence of costimulation becomes anergic and may undergo apoptosis (3-6), blocking the delivery of costimulatory signals at the time of transplantation has proven to be an effective strategy to prevent allograft rejection and facilitate tolerance induction at least in small animal models.

One key costimulatory pathway involves the interaction between the T cell associated CD40 ligand (CD154) molecule with CD40 on the APC (7). The broad distribution of this pathway has made blockade of CD40L signaling an attractive target in experimental transplant models. Several reports demonstrate that treatment with anti-CD40L antibodies can prolong the survival of allografts, including renal and islet grafts in nonhuman primate models (8-12). Moreover, the combination of anti-CD40L treatment with non-myeloablative conditioning strategies has been shown to induce high levels of prolonged mixed allogeneic chimerism and lead to robust tolerance in both rodent and primate models of transplantation (13-20). While a number of reports have demonstrated that CD40L blockade can effectively inhibit $CD4^+$ T cell responses (7, 21, 22), further studies investigating the mechanisms of this pathway suggest that it has important implications in several mechanisms of tolerance. For instance, in a model involving donor specific transfusion and CD40L blockade, tolerance to islet allografts is achieved through the maintenance of T cell anergy, which is dependent upon CTLA-4 negative signaling (23, 24). Others have demonstrated that CD40L blockade, combined with anti-CD8 mAb, involves the amplification of regulatory mechanisms as the primary mechanism of tolerance (25, 26). Moreover, when both CD40L and CD28 signaling are inhibited, long-term acceptance of allografts is mediated through apoptotic deletion of alloreactive T cells (27, 28). Recently, selective depletion of activated T cells by complement- and Fc receptor-mediated mechanisms, as opposed to costimulation blockade, have been implicated in promoting allograft survival by anti-CD40L treatment (29). Taken together, these studies emphasize the diversity of this molecule in influencing immune responses, and raise questions as to the relative contributions of anergy, regulation, and deletion, either as a consequence of costimulation blockade or other mechanisms, in mediating tolerance in these models.

In recent years, through the identification of novel costimulatory and coinhibitory pathways, we have learned to appreciate that many other molecules may act to influence T cell responses. One such molecule is inducible costimulator (ICOS), a member of the CD28 superfamily important in T cell activation, splenic germinal center formation and immunoglobulin class switching (30-34). ICOS is induced on both CD4$^+$ and CD8$^+$ T cells after CD28 signaling (35), and interaction with its ligand B7RP-1 (B7h, GL50, LIOCS), leads to the delivery of signals that regulate both Th1 and Th2 cell differentiation (31, 36-40). A series of studies have demonstrated that ICOS functions as a critical costimulatory pathway in allograft rejection, where blockade of ICOS signaling is synergistic with other costimulatory blocking agents, or conventional immunosuppression, in facilitating long-term graft acceptance and impairing chronic allograft rejection (41-47).

In previous work, we have shown that combination therapy with anti-ICOS and anti-CD40L mAbs results in indefinite islet allograft acceptance across a fully-MHC mismatched barrier in all recipients (43). In this study, we confirm our results and further explore the mechanisms of tolerance in this model, highlighting that tolerance is not merely an absence of functional allospecific T cells, but an active regulation of the alloimmune response. Moreover, we report on the effect of this strategy in controlling the autoimmune process of diabetes since successful tolerance protocols in islet transplantation must contend with both alloimmune and autoimmune processes of graft rejection.

6.2 MATERIALS AND METHODS

6.2.1 ANIMALS

Adult C57BL/6 (H-2^b), BALB/c (H-2^d), and CBA/JCr (H-2^k) mice were obtained from Charles River Canada. Immunodeficient C57BL/6-RAG1-KO male mice were originally purchased from Jackson Laboratories (Bar Harbor, Maine) and bred in-house. Female NOD mice were obtained from Taconic Canada at 4 weeks of age. C57BL/6, BALB/c, CBA/JCr mice were housed under standard conditions, while C57BL/6-RAG-KO and NOD mice were housed under specific pathogen-free conditions. All animals were cared for in accordance with the guidelines established by the Canadian Council on Animal Care.

6.2.2 INDUCTION OF DIABETES AND ISLET TRANSPLANTATION

C57BL/6 and immunodeficient C57BL/6-RAG-KO recipient mice were rendered chemically diabetic by a single injection of streptozotocin (200 mg/kg i.v., Sigma-Aldrich, Canada). Donor islets were isolated from fully MHC-mismatched BALB/c mice by collagenase digestion (1 mg/ml, Sigma-Aldrich) followed by Ficoll purification (48, 49) (Sigma-Aldrich). Approximately 500 islets were transplanted under the left renal capsule of diabetic recipient mice. Allograft function was monitored by serial blood glucose measurements. Successful engraftment was defined by correction of serum glucose level to <8 mmol/L by the third day post-transplant, and graft rejection was defined as a rise in serum glucose >15 mmol/L for two consecutive days.

6.2.3 REAGENTS AND TREATMENT PROTOCOLS

Anti-CD40L mAb (MR1) was purchased from BioExpress (West Lebanon, NH), and production and characterization of the non-depleting anti-ICOS mAb (12A8) has been described previously (41). An isotype-matched IgG2b control mAb was also obtained from BioExpress. Recipients of islet allografts were treated with anti-CD40L mAb (0.25 mg, days 0, 2, 4 and 6) and/or anti-ICOS mAb (0.1 mg/d x 14 days) beginning on the day of transplantation. Female NOD mice were treated with an IgG2b control mAb (0.1 mg/d x 14 days) or anti-CD40L mAb (0.25 mg, days 0, 2, 4, 6 and 10) and/or anti-ICOS mAb (0.1 mg/d x 14 days). Treatment in NOD mice was initiated at 10 weeks of age, during development of insulitis but prior to the onset of spontaneous diabetes. All reagents were administered i.p.

6.2.4 MIXED LYMPHOCYTE REACTIONS (MLRS)

Splenocytes were cultured in duplicate wells containing $2x10^5$ responder cells with various dilutions of irradiated (1500 Rad) stimulator cells. Responder cells were obtained from naïve and long-term engrafted mice, while stimulator cells were derived from C57BL/6 (syngeneic), BALB/c (donor) and CBA/J (3rd party) mice. After culture at 37 °C for 3 days, cells were pulsed for 18 h with 1 μCi ^{3}H thymidine/well, harvested and thymidine incorporation determined. To assess the presence of regulatory T cells in tolerant mice, splenocytes from tolerant and naïve mice were co-cultured in MLRs to donor antigen. The ratio of tolerant to naïve cells in co-culture reactions was increased to ensure that adequate regulatory cell numbers were present to detect a potential suppressive effect. Additional MLRs with only naïve and tolerant cells were included to control for changing total cell number and to assess the proliferation of each cell population individually.

6.2.5 CYTOTOXIC LYMPHOCYTE REACTIONS (CTLS)

Cytotoxic responses were assayed by the JAM Test as previously described (50). Briefly, $5x10^6$ spleen cells from C57BL/6 naïve and tolerant mice (responders) were stimulated for 5 days with $2x10^6$ irradiated BALB/c (donor) spleen cells. Con A blast targets were set up 40 hours prior to the CTL assay by culturing $1.5x10^6$ naïve syngeneic and BALB/c spleen cells with Con A (1.25 μg/ml), then labeling with ^{3}H-thymidine. Lysis of target cells was tested at various responder to target ratios.

6.2.6 IMMUNIZATION OF TOLERANT MICE WITH DONOR SPLEEN CELLS

Donor spleen cells were prepared by making a single cell suspension from harvested spleens of BALB/c mice in PBS, followed by passage of the cell suspension through a nylon mesh. Viable cells were counted and adjusted to 20×10^6 cells/ml. Long-term engrafted mice previously treated with anti-ICOS and -CD40L mAbs were subsequently immunized with $5x10^6$ spleen cells (0.25ml) i.p. and then followed for graft rejection.

6.2.7 CONFIRMATION OF GRAFT FUNCTION AND RE-TRANSPLANTATION

Long-term graft function in mice maintaining normoglycemia beyond 100 days was confirmed by a return to hyperglycemia following nephrectomy of the kidney bearing the islet graft. To test for donor specific immunological tolerance, nephrectomized mice underwent re-transplantation of same donor-strain (BALB/c) or third party (CBA/J) islets into the remaining contralateral kidney. No immunosuppressive therapy was given and blood glucose was monitored serially to detect graft rejection.

6.2.8 ISLET-KIDNEY COMPOSITE GRAFT TRANSPLANTATION

C57BL/6 mice treated with anti-ICOS and -CD40L mAbs and C57BL/6-RAG-KO immunodeficient mice underwent nephrectomy of their islet-bearing kidneys after long-term maintenance of normoglycemia beyond 100 days. The islet (BALB/c) - kidney (C57BL/6) composite grafts from both groups of mice were subsequently transplanted into streptozotocin-treated, diabetic, naïve C57BL/6 mice. Renal transplantation was performed as previously described (51) with the donor renal artery and vein being anastomosed to the recipient aorta and inferior vena cava, respectively. An anastomosis between the donor ureter and recipient bladder was also created. Blood glucose in mice receiving islet-kidney composite grafts was serially monitored to detect for rejection of the alloislet graft within the transplanted kidney.

6.2.9 IMMUNOPATHOLOGY

Following nephrectomy of graft-bearing kidneys, one half of the islet allograft was embedded in Histo Prep (Fisher Scientific, Canada) and frozen in liquid nitrogen for storage at –70 °C until sectioning. The other half of the graft was stored in formalin and then paraffin embedded. Cryostat sections were stained by immunoperoxidase (41) using mAbs to CD4 and CD25 or isotype controls (Pharmingen, San Diego, CA) and rabbit anti-mouse *Foxp3* or control IgG. Paraffin sections were stained with hematoxylin and eosin to assess overall cellularity.

6.2.10 STATISTICAL ANALYSIS

Graft survival was analyzed using the Kaplan-Meier method, and statistical comparisons among groups were performed using the log rank test (SPSS version 10.0, Chicago, IL).

6.3 RESULTS

6.3.1 COMBINATION THERAPY WITH ANTI-ICOS AND ANTI-CD40L MABS INDUCES LONG-TERM ISLET ALLOGRAFT SURVIVAL

Our first objective was to determine the effect of costimualtion blockade of ICOS and CD40L signaling on islet allograft survival. Diabetic C57BL/6 mice were transplanted with approximately 500 fully-MHC mismatched BALB/c islets under the renal capsule and treated with anti-ICOS and -CD40L mAbs, either alone or in combination. Compared to untreated control mice, anti-ICOS mAb treatment alone did not prolong graft survival (MST=13 days), while CD40L monotherapy resulted in significant prolongation of graft survival with 60% (6/10) of grafts surviving > 100 days. Combination therapy with anti-ICOS mAb and CD40L mAb resulted in the most potent prolongation of graft survival, with 93% (26/28) of grafts being maintained > 100 days (Figure 6-1). This level of acceptance was significantly higher than that achieved with individual monotherapy approaches ($p<0.05$).

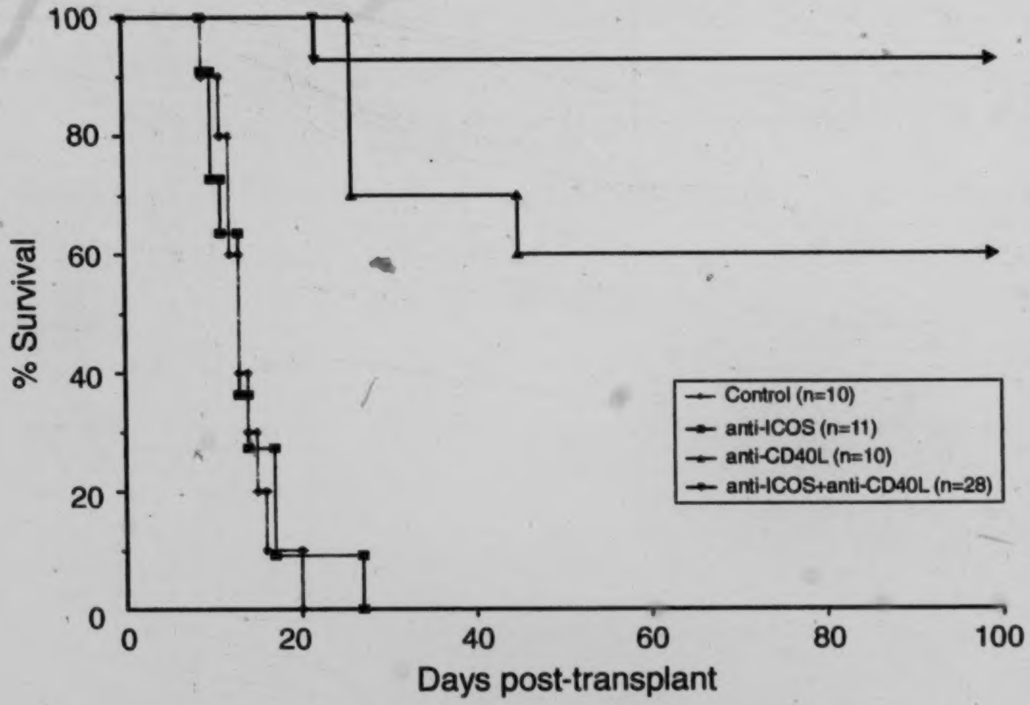
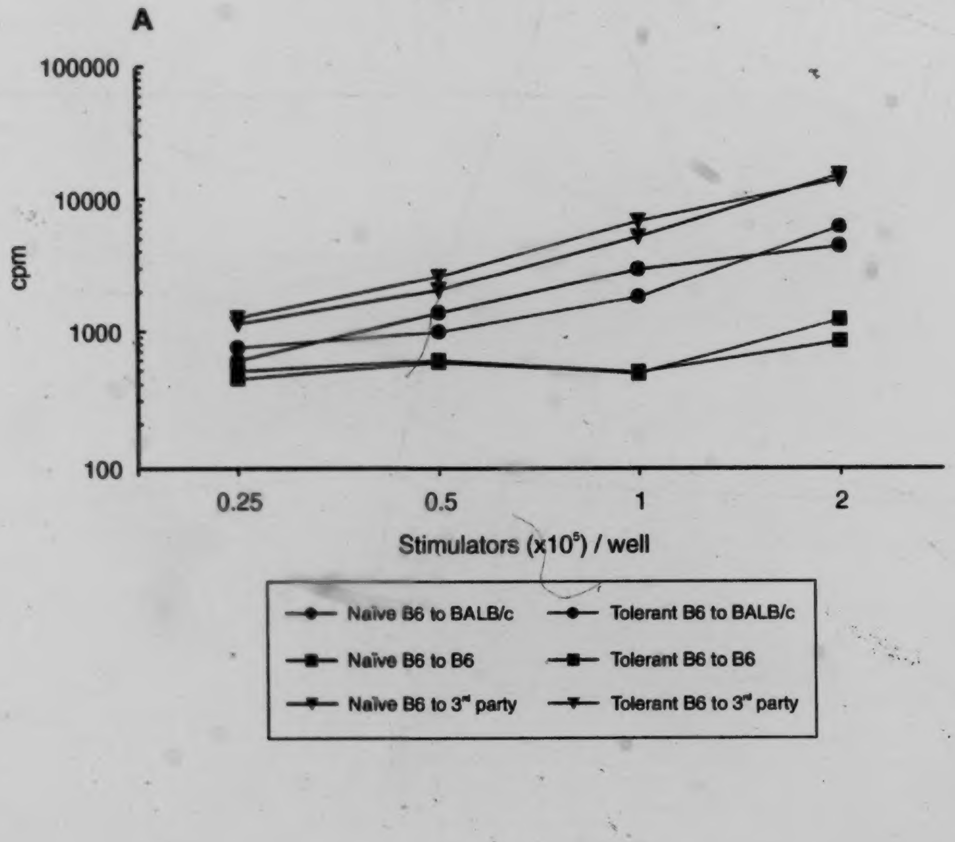


Figure 6-1: Islet allograft survival under anti-ICOS and/or anti-CD40L therapy. Simultaneous blockade of ICOS and CD40L signaling results in a significant level of long-term allograft survival, which is not accomplished through individual monotherapies.

6.3.2 LONG-TERM ENGRAFTED MICE TREATED WITH COMBINATION THERAPY DEMONSTRATE INTACT ANTI-DONOR RESPONSES IN VITRO

In vitro T cell responses were analyzed in mice maintaining normoglycemia beyond 100 days after initial combination therapy with anti-ICOS and anti-CD40L mAbs. Anti-donor proliferative and cytotoxic responses were evaluated in long-term engrafted mice and compared to responses from naïve mice. Standard mixed lymphocyte reactions to both donor (BALB/c) and third party (CBA/J) antigen (Figure 6-2A), and cytotoxic lymphocyte reactions to donor antigen (Figure 6-2B) were assayed. No significant difference in either proliferative or cytotoxic responses between lymphocytes from long-term engrafted mice or naïve mice was detected. These results indicate that although mice treated with combination therapy maintain long-term allograft function, they do not demonstrate long-term unresponsiveness to donor spleen cells in vitro. One possibility was that the grafts are maintained on the basis of immunological ignorance (52). To test this theory, we immunized long-term engrafted mice with 5×10^6 donor spleen cells as an immunological challenge to precipitate rejection. After immunization, all islet allografts (n=5) continued to maintain normal function beyond 50 days, as evidenced by sustained normoglycemia (data not shown), suggesting that long-term allograft acceptance is not merely based on a mechanism of ignorance.



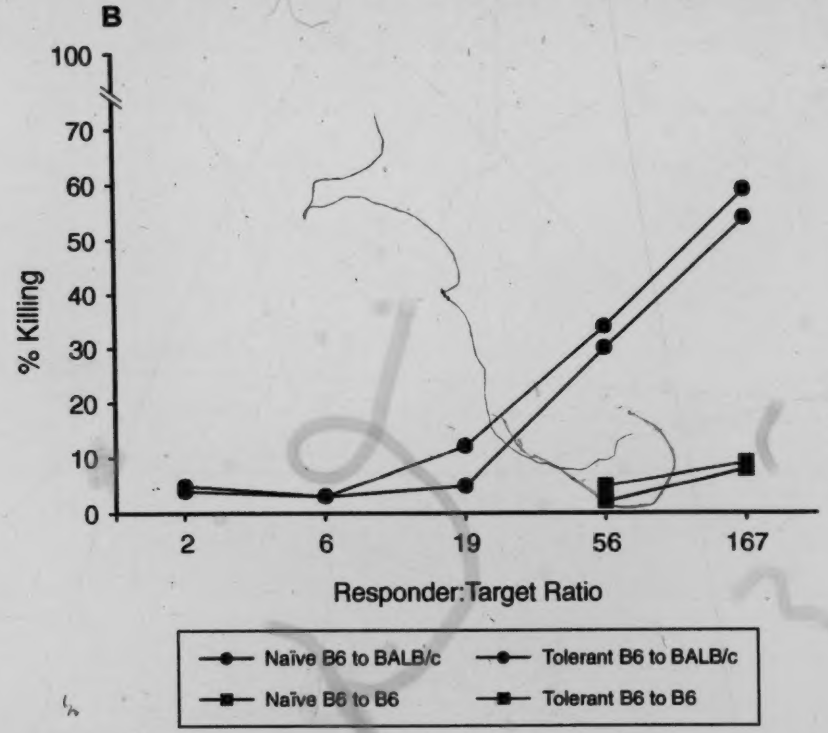


Figure 6-2: In vitro responses in long-term engrafted mice treated with anti-ICOS and anti-CD40L mAbs.
Lymphocytes from long-term engrafted mice maintained robust anti-donor proliferative (A) and cytotoxic (B) responses comparable to naïve responses.

6.3.3 LONG-TERM ENGRAFTED MICE TREATED WITH COMBINATION THERAPY DEMONSTRATE DONOR-SPECIFIC IMMUNOLOGICAL TOLERANCE IN VIVO

Although immunization with donor spleen cells did not trigger allograft rejection, it remained possible that recipients were in fact "ignorant" of donor antigen but unable to reject a well-established graft, which may not deliver the necessary signals needed to recruit effector T cells to the graft site (53). To more fully distinguish whether graft acceptance was due to immunological ignorance or tolerance, we challenged long-term engrafted recipients with a second same donor islet allograft.

Long-term allograft acceptance (>100 days) was first confirmed in mice treated with anti-ICOS and anti-CD40L mAbs by nephrectomy of the graft-bearing kidney, which resulted in a prompt return of hyperglycemia in all cases (n=11). These mice were subsequently re-challenged with a second same donor (BALB/c) islet allograft in the remaining contralateral kidney without further immunosuppressive therapy. All second same donor allografts were accepted long-term (>100 days), indicating that treatment with anti-ICOS and anti-CD40L mAbs induces indefinite allograft acceptance on the basis of immunological tolerance and not ignorance. Another cohort of tolerant mice was then re-challenged with third party (CBA/J) islet allografts, which were rapidly rejected, indicating that tolerance in this model is donor-specific (Figure 6-3).

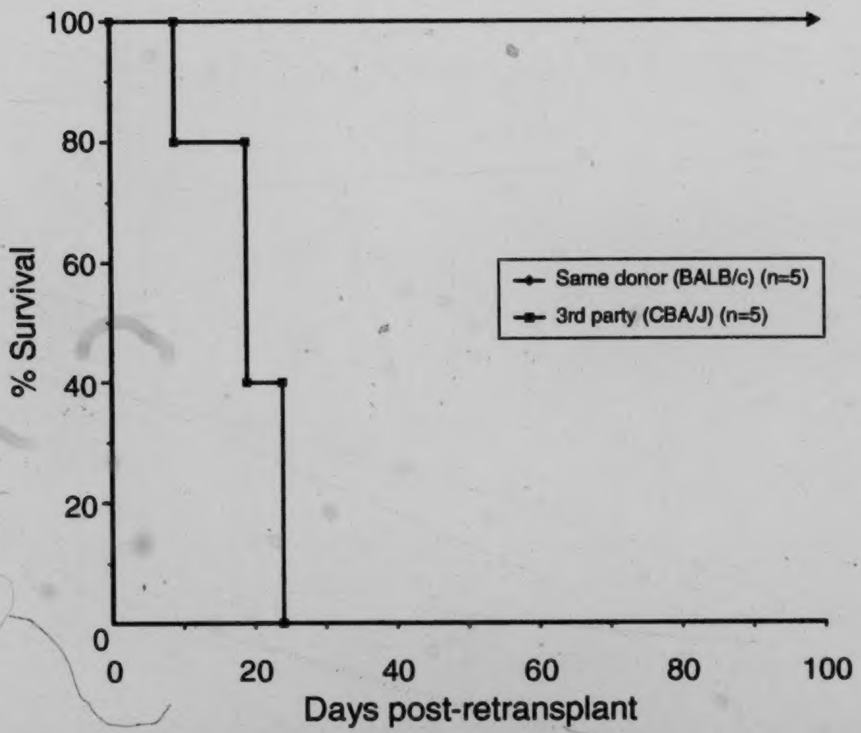


Figure 6-3: Long-term engrafted mice treated with anti-ICOS and anti-CD40L therapy demonstrate donor-specific tolerance in vivo

6.3.4 TOLERATED ALLOGRAFTS FROM MICE TREATED WITH COMBINATION THERAPY DEMONSTRATE THE PRESENCE OF INTRA-GRAFT T CELLS WITH REGULATORY PHENOTYPE

We next sought to address the issue of long-term maintenance of immunological tolerance in vivo despite the presence of strong anti-donor proliferative and cytotoxic responses in vitro. We hypothesized that long-term allograft acceptance in our model is dependent on a dominant immunoregulatory mechanism that can actively constrain alloaggressive T cell responses. The role of specific regulatory T cells that can co-exist in the presence of robust alloreactivity and maintain peripheral allograft tolerance has been well-described, and their function can be assessed in vitro since they can be isolated from spleens of tolerant mice (54-56). Given that splenic lymphocytes from tolerant mice generated robust proliferative responses to donor antigen in vitro (Figure 6-2A), it appeared unlikely that regulatory T cells may be present in the spleens of these mice. To more fully rule out this possibility, a standard MLR assay was used to detect for the presence of donor specific regulatory T cells. Lymphocytes from tolerant mice were co-cultured with lymphocytes from naïve mice to determine if allo-specific proliferation could be reduced with increasing numbers of lymphocytes from tolerant mice (56). As expected, the ability of naïve T cells to proliferate in response to donor antigen was not reduced in the presence of lymphocytes from tolerant mice, at any given concentration (data not shown). Furthermore, additional co-culture MLRs were performed where the proportion of naïve:"tolerant" lymphocytes was serially increased, while keeping the total number of cells in each well constant. Even with this technique, the MLR to donor-antigen remained strong throughout (data not shown). Therefore, the results from these assays suggest that donor specific regulatory T cells are not present in the spleens of mice treated with anti-ICOS and anti-CD40L mAbs. However, it is possible that the specificity or effector function of these cells is restricted to islet antigen alone and not donor hematopoeitic cells, rendering them functionally undetectable in vitro.

In recent years, CD4$^+$ regulatory T cells have also been identified within the grafts of tolerant mice and are capable of mediating a dominant suppressive effect on naïve T cells (57). Therefore, a regulatory mechanism restricted to the graft site may be operating in long-term engrafted mice treated with anti-ICOS and anti-CD40L mAbs. Since CD4$^+$CD25$^+$ T cells have been well-described as critical mediators in the maintenance of peripheral allograft tolerance (58, 59), we performed immunoperoxidase staining for CD4 and CD25 in tolerated grafts. In addition, staining for the novel transcription factor *Foxp3*, a key gene in the development of regulatory T cells, was also performed to specifically identify the presence of T cells with regulatory function (60). *Foxp3* cannot be induced in naïve T cells by activation, in contrast to cell surface molecules such as CD25, GITR and CTLA, therefore, it serves as a specific molecular marker for regulatory T cells (60). As depicted in Figure 6-4, tolerated islet grafts from anti-ICOS and anti-CD40L treated mice demonstrated marked peri-islet staining for CD4, of which a significant number also expressed CD25. Interestingly, discernable peri-islet staining was also demonstrated for *Foxp3*. Expression of theses markers was not seen in healed-in allografts from untreated, immunodeficient C57BL/6-RAG-KO mice. These observations suggest that the maintenance of tolerance in mice treated with anti-ICOS and anti-CD40L mAbs may be dependent on a dominant regulatory mechanism mediated by intra-graft regulatory T cells.

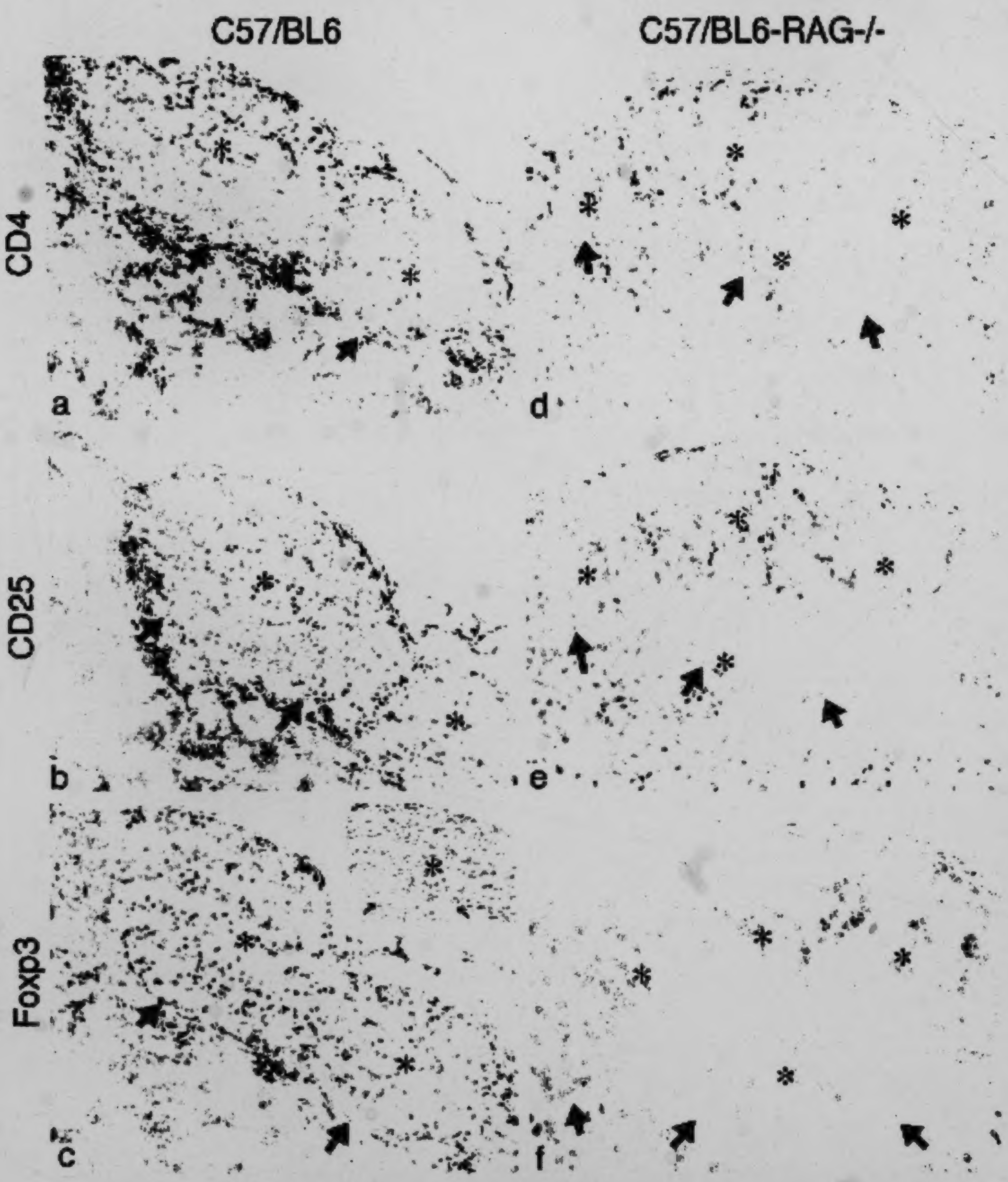


Figure 6-4: Immunohistology of tolerated grafts (>100 days) from anti-ICOS + anti-CD40L treated recipients suggests the presence of intra-graft regulatory T cells. Immunoperoxidase staining of BALB/c islet allografts in anti-ICOS/CD40L mAb-treated wild-type recipients demonstrates peri-islet expression of CD4, CD25, and Foxp3 (a-c), suggesting the presence of intra-graft T cells with regulatory phenotype. Untreated RAG B6 recipients did not demonstrate any appreciable staining for these markers (d-f). Asterisks indicate well-preserved islets, arrows indicate boundary of kidney vs. islet grafts, and inset in (c) shows lack of staining with Foxp3-peptide absorbed antibody (x300 magnification, representative of 4 grafts/group).

6.3.5 INTRA-GRAFT LYMPHOCYTES IN TOLERATED ISLET ALLOGRAFTS CAN MEDIATE DOMINANT TOLERANCE

As an in vivo functional test for intra-graft mediated dominant tolerance, we performed a re-transplantation procedure involving the transfer of tolerated islet allografts from long-term engrafted mice into naïve mice. Specifically, the islet-bearing kidneys from tolerant mice previously treated with anti-ICOS and anti-CD40L were explanted, and this islet (BALB/c) - kidney (C57BL/6) composite graft was subsequently re-transplanted into streptozotocin-treated naïve C57BL/6 mice. To serve as controls, islet (BALB/c) – kidney (C57BL/6) composite grafts from streptozotocin-treated C57BL/6-RAG-KO mice were also harvested after long-term maintenance of normoglycemia (>50 days) and subsequently re-transplanted into streptozotocin-treated naïve C57BL/6 mice (Figure 6-5). Blood glucose in transplanted mice was monitored serially to detect for graft rejection. As shown in Figure 6-6, islet allografts from both long-term engrafted (>100 days) treated and immunodeficient recipients were well preserved prior to re-transplantation. However, while grafts from treated recipients were accepted long-term and remained preserved in naïve mice, grafts from control immunodeficient mice were rejected at a median of 13 days (Figure 6-6 and Figure 6-7). Moreover, expression of CD4, CD25, and *Foxp3* on donor allografts from tolerant treated mice was maintained long-term (>100 days) after re-transplantation into naive mice (Figure 6-8).

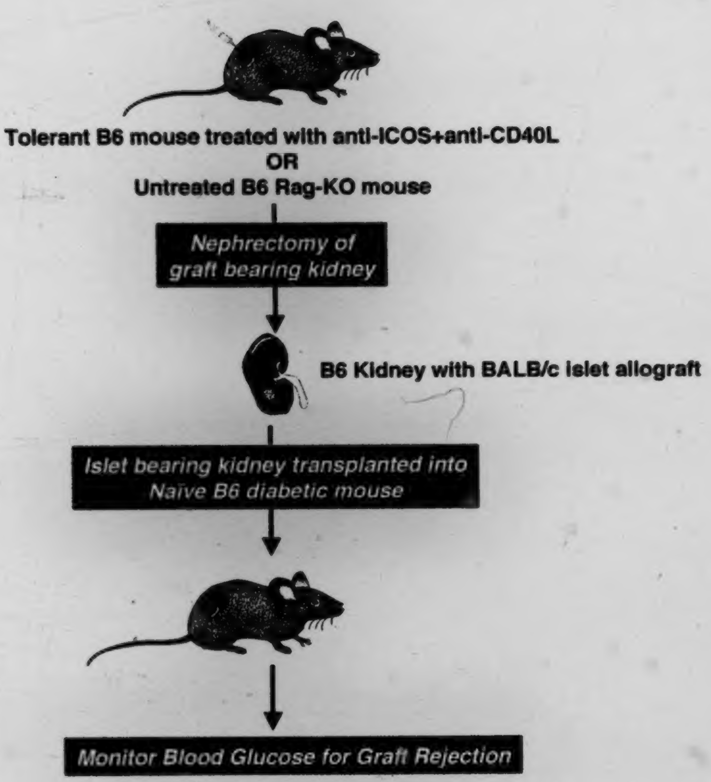

Tolerant B6 mouse treated with anti-ICOS+anti-CD40L
OR
Untreated B6 Rag-KO mouse
Nephrectomy of graft bearing kidney
B6 Kidney with BALB/c islet allograft
Islet bearing kidney transplanted into Naïve B6 diabetic mouse
Monitor Blood Glucose for Graft Rejection

Figure 6-5: In vivo functional test for dominant regulatory tolerance.
Tolerated islet allografts from either anti-ICOS+anti-CD40L treated or immunodeficient recipients were transferred into sreptozotocin-treated naïve recipients and followed for graft rejection.

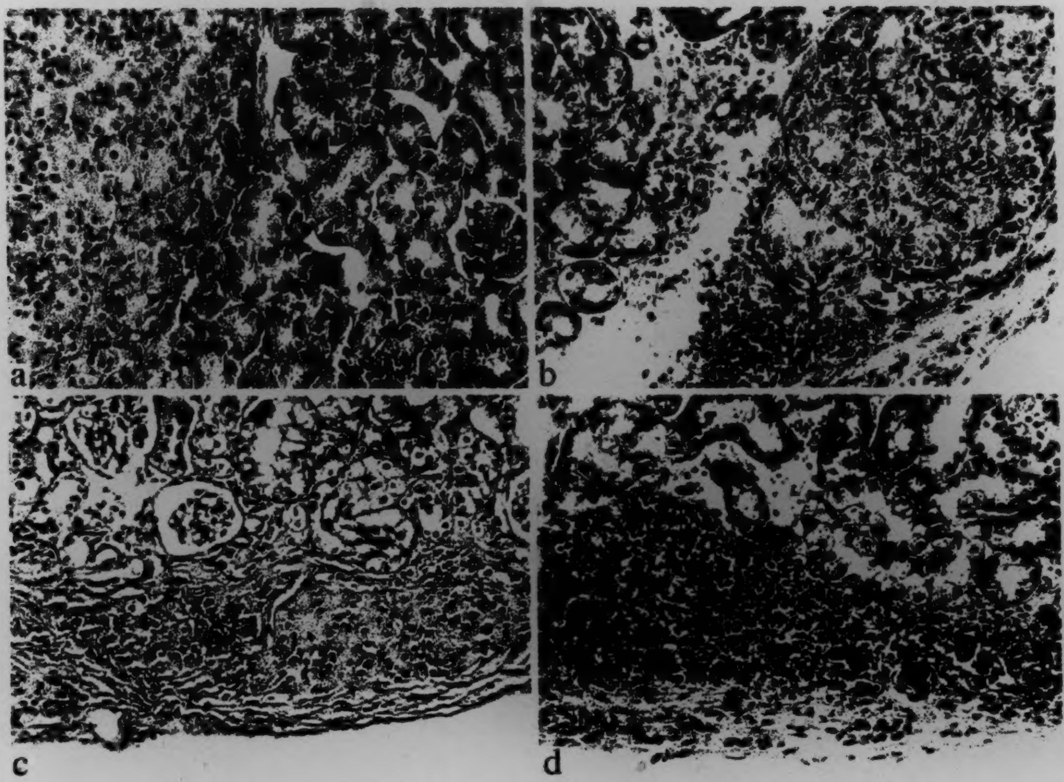


Figure 6-6: Histology of islet allografts (x250 magnification)

a) Well preserved islet allograft post anti-ICOS+anti-CD40L mAb therapy (>100 days)
b) Well preserved islet allograft in naïve recipient post re-transplantation (>100days)
c) Well preserved islet allograft in B6-RAG recipient (>100 days)
d) Acute rejection of islet allograft at day 13 following re-transplantation from RAG donor (>100 days) into naïve recipient

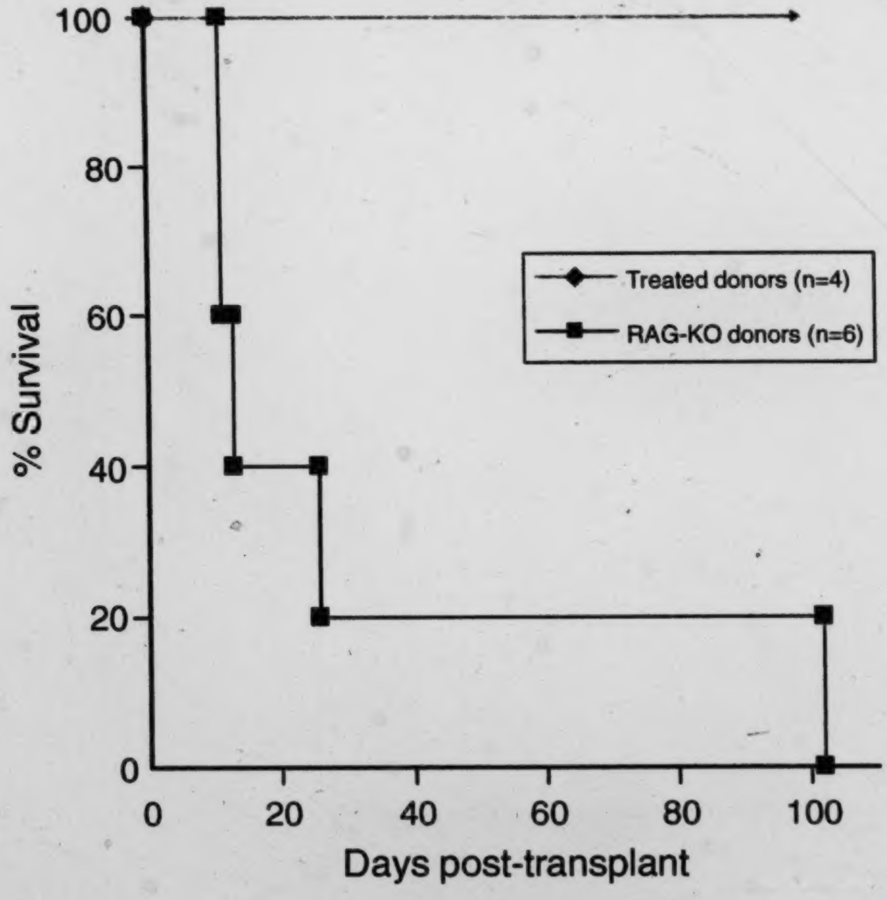


Figure 6-7: Re-transplanted islet allograft survival in naïve recipients
Islet allograft from treated donors, bearing intra-graft T cells of regulatory phenotype, survive indefinitely in naïve recipients, while grafts from RAG-KO mice are rejected

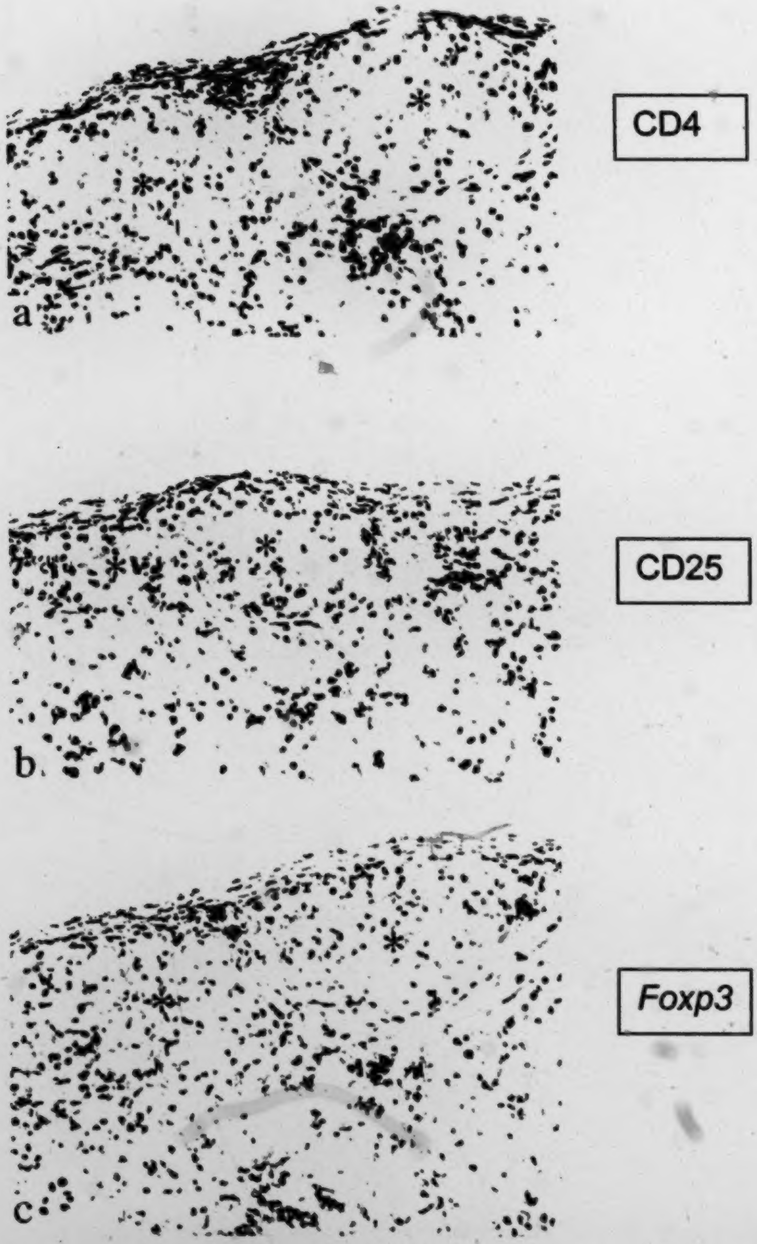


Figure 6-8: Immunohistology of re-transplanted islet allograft from a treated donor into a naïve recipient (>100 days).
Prominent peri-islet staining for CD4 (a) and CD25 (b) with discernable expression of *Foxp3* (c) is maintained long-term in islet allografts after re-transplantation from tolerant treated mice into naïve recipients. Asterisks indicate well preserved islet tissue (x300 magnification).

The rejection of the control islet grafts from immunodeficeint mice indicates that it is not the healed in characteristics of the graft or the fact that it has been "parked" that allows for long-term acceptance of islet grafts from tolerant treated mice. These results indicate that treatment with anti-ICOS and anti-CD40L mAbs leads to the establishment of intra-islet graft regulatory lymphocytes with the capacity to protect the allograft against anti-donor responses through dominant tolerance.

6.3.6 COMBINATION THERAPY WITH ANTI-ICOS AND ANTI-CD40L MABS PREVENTS THE ONSET OF SPONTANEOUS AUTOIMMUNE DIABETES IN NOD MICE

Successful tolerance protocols in islet transplantation must be effective not only at preventing alloimmune rejection, but also at overcoming the underlying autoimmune process of diabetes. Therefore, having demonstrated that combination therapy with anti-ICOS and anti-CD40L can prevent alloimmune rejection of islet grafts, we proceeded to test the effectiveness of this therapy in preventing the autoimmune destruction of islet cells. This was accomplished by treating female NOD mice with anti-ICOS and anti-CD40L mAbs, either alone or in combination for 14 days, beginning at 10 weeks of age. Female NOD mice in our colony develop pancreatic islet infiltration by leukocytes beginning at about 6-7 weeks of age. Without any form of immunomodulation, approximately 50% of mice develop diabetes by 25 weeks of age, progressing to 75% by 33 weeks. Treatment of NOD mice with an IgG2b control mAb did not significantly alter the rate of diabetes as compared to untreated mice (Figure 6-9A). Monotherapy with either anti-ICOS mAb or anti-CD40L mAb resulted in a marked, but non-significant reduction in the onset diabetes compared to mice treated with control mAb (37% and 35% versus 63%, respectively, $p>0.05$). The combination of anti-ICOS mAb with anti-CD40L mAb led to a more potent reduction in the onset of diabetes with only 11% (2/19) of mice becoming diabetic ($p=0.065$ compared to individual monotherapies; $p< 0.001$

compared to control mAb treated) (Figure 6-9B). Taken together, these results indicate that dual blockade of ICOS and CD40L signaling is highly effective at preventing alloimmune rejection and autoimmune destruction of islet cells.

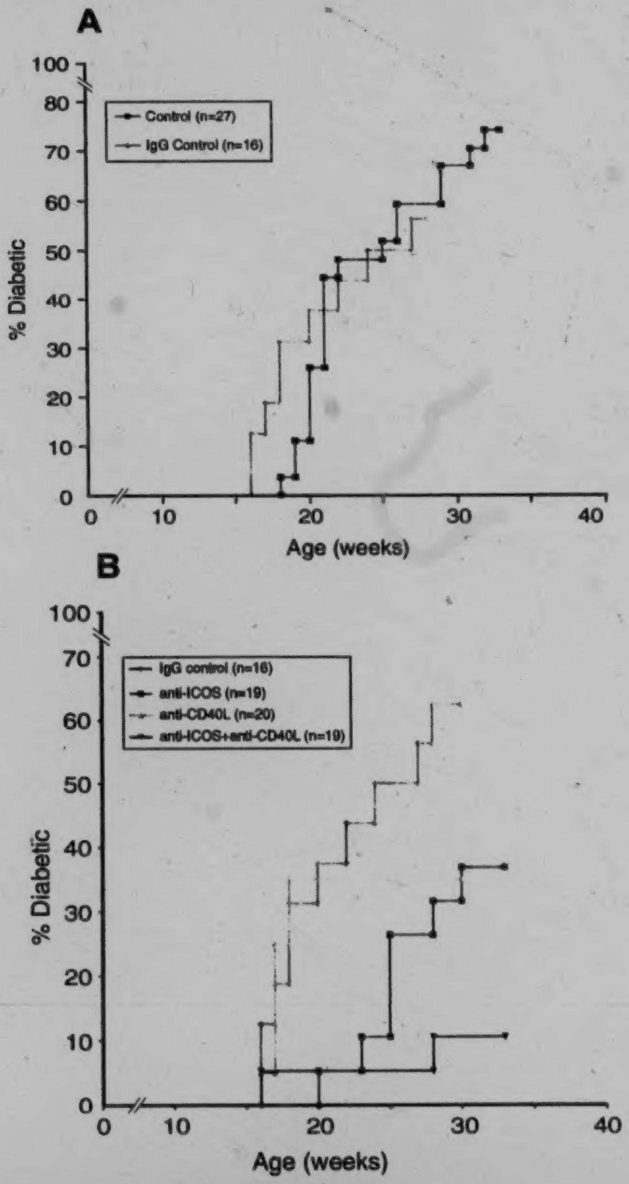


Figure 6-9: Treatment with anti-ICOS and/or anti-CD40L prevents the onset of spontaneous autoimmune diabetes in NOD mice.
While monotherapy with either anti-ICOS mAb or ani-CD40L mAb decreased the onset of diabetes, the combination of these agents was significantly more potent, with only 11% (2/19) of NOD mice becoming diabetic.

6.4 DISCUSSION

The blockade of costimulation is widely accepted as a potent strategy to promote long-term allograft acceptance and, in some instances, to induce immunologic tolerance. However, with the ongoing identification of novel costimulatory molecules, it is apparent that T cell activation and allograft rejection can occur despite blockade of a single costimulatory pathway. This has been supported by reports of allograft rejection in mice deficient in CD28 or CD40L signaling, where rejection is thought to be due to T cell activation through alternative costimulatory pathways (61-63). Moreover, the efficacy of costimulation blockade in regulating alloimmune responses is organ/tissue dependent, with reduced success in more stringent fully allogeneic models such as skin and islet transplantation (64). It has been shown that approximately 6000-fold less alloreactive T cells are required to reject islet allografts compared to cardiac allografts, rendering islets significantly more susceptible to rejection (65). Furthermore, while renal allografts in nonhuman primates can survive for greater than 1 year after discontinuation of anti-CD40L treatment, islet allografts are rapidly rejected within several months (9, 10). These observations suggest more robust strategies that combine two or more agents may be required to induce tolerance in islet transplantation. The results from this study support this concept by demonstrating that blockade of ICOS and CD40L signaling is significantly more effective in facilitating islet allograft acceptance when used in combination than as individual monotherapies.

The need for combined strategies to achieve long-term islet allograft survival has been validated in prior studies involving ICOS and CD40L blockade. For instance, while anti-ICOS monotherapy has been shown to provide some protection to heart and liver allografts from rejection (42, 46), it is not effective at promoting islet graft acceptance (66). However, when anti-ICOS is combined with simultaneous blockade of CD28-B7 signaling, tacrolimus therapy or rapamycin treatment, survival of islet allografts is significantly improved (43, 47). Similarly, while blockade of CD40L signaling alone is not effective at inducing tolerance in stringent models of islet and skin allotransplantation, adjunctive strategies such as donor specific transfusion (67, 68), CD45 signaling blockade (69), blockade of the adhesion/homing receptor LFA-1 (70) or concurrent stimulation of negative signaling through Programmed cell death 1 (PD-1) (71), can all lead to indefinite islet allograft acceptance. These reports demonstrate that combination strategies with either anti-ICOS or anti-CD40L have considerable tolerogenic potential in islet transplantation. It is not surprising therefore that the combination of these agents in this study results in a high level of long-term islet allograft survival.

The synergy between ICOS and CD40L blockade may be related to the complementary inhibition of both CD4$^+$ and CD8$^+$ T cell responses, based on the differential effects of these molecules on effector T cell function. While inhibition of CD40L signaling can effectively prevent donor specific CD4$^+$ T cell responses, it has no appreciable effect on the function of alloreactive CD8$^+$ T cells (72, 73). As a consequence, several reports have indicated that rejection can still occur despite blockade of CD40L signaling due to alloreactive CD8$^+$ T cells that are resistant to costimulation blockade (25, 74, 75). Therefore, in stringent models, strategies to induce tolerance through CD40L blockade require adjuncts that can provide direct anti-CD8+ T cell activity. This has been validated in studies where the efficacy of CD40L blockade is dramatically enhanced by concurrent treatment with anti-CD8 or CTLA4-Ig, both of which promote the deletion of CD8$^+$ T cells (25-28, 74). In our model, combination with anti-ICOS may be providing a similar effect based on evidence that ICOS plays a critical role in regulating both antigen-specific CD4$^+$ and CD8$^+$ T cell responses (35, 76, 77). Moreover, blockade of ICOS signaling in transplantation models has been shown to effectively control the expansion and differentiation of both effector T cell compartments, thereby facilitating acceptance of the allograft (43, 46). Importantly, while the generation and/or maintenance of allospecific CD8$^+$ T cells is contingent on ICOS signaling, this effect is independent of early CD4$^+$ T cell help (46). Therefore, the role of ICOS in CD8$^+$ T cell responses makes it a suitable complementary therapy to enhance of CD40L blockade. Its benefit is of particular interest in islet transplantation given that CD8$^+$ cells have been reported to be important effectors of rejection in murine models of islet transplantation (78).

Having demonstrated the efficacy of combined ICOS and CD40L blockade, we next sought to determine the strength of tolerance induced in this model. We evaluated T cell responses in long-term engrafted mice in MLR and CTL assays and found that tolerant recipients exhibit potent proliferative and cytotoxic reactivity to donor alloantigen. These findings indicate that complete clonal deletion of donor-reactive T cells is not entirely required for the long-term acceptance of islet allografts after combined anti-ICOS and anti-CD40L therapy. A possible mechanism may be the induction of immunological ignorance in tolerant mice, where a well-established graft can be maintained despite the presence of detectable alloreactive T cells. To determine if tolerant mice were "'ignorant" of their allografts, immunizations with donor type splenocytes were performed in long-term engrafted mice. This has been reported as an appropriate immunological challenge to precipitate the rejection of islet grafts that are maintained on the basis of immunological ignorance (79, 80). After immunization, all mice sustained normal allograft function beyond 50 days, suggesting that permanent allograft acceptance is not dependent on immunological ignorance. However, since a well-established (healed-in) allograft may be refractory to rejection despite successful immunization (79), we challenged long-term engrafted mice with a second same donor islet allograft to more thoroughly rule out a mechanism of ignorance and to test for immunological tolerance.

Long-term engrafted mice underwent nephrectomy of their graft-bearing kidneys to confirm the functionality of the islet graft, followed by re-challenge with a second same donor or third party islet graft. These mice accepted same donor but promptly rejected third party allografts, indicating that anti-ICOS and anti-CD40L treatment induces a state of donor specific tolerance. Therefore, while tolerance is established, the long-term maintenance of allograft function continues despite the presence of detectable alloreactive T cells. One possibility for these divergent findings may be attributed to the generation of split tolerance, where the recipient may be tolerant to the islet allograft but not to donor splenocytes (81). Another possible explanation is that tolerance in this model is dependent on an active, dominant regulatory mechanism that controls peripheral alloreactivity, thereby permitting long-term allograft function.

While deletional mechanisms play an essential role in the induction of tolerance through costimulation blockade, the maintenance of tolerance has been proposed to be critically dependent upon immunoregulatory mechanisms that actively restrain alloreactive T cell responses (82-84). Regulation can co-exist with the presence of donor reactivity and provides a mechanism to contend with both alloreactive T cells that have escaped deletion and the continual emigration of new donor specific T cells from the recipient thymus. Several studies have implicated a regulatory mechanism in the maintenance of tolerance in strategies involving CD40L blockade (23, 25, 26, 70, 73, 85) and ICOS blockade (46). Moreover, studies in STAT4$^{-/-}$ (impaired Th1 response) and STAT6$^{-/-}$ (impaired Th2 response) models demonstrate that both ICOS and CD40L blockade are less effective under conditions that favour Th1 responses in mice (in STAT6$^{-/-}$ mice), further suggesting the importance of regulatory mechanisms in enabling long-term graft acceptance (46, 86). Therefore, while deletion and/or anergy may be important in the induction of tolerance in this model, the long-term maintenance of tolerance may be mediated by regulation. This hypothesis is consistent with our findings of a lack of permanent deletion and the presence of functional alloreactivity in mice maintaining long-term allograft function.

Regulatory CD4$^+$CD25$^+$ T cells have been characterized as key mediators in the maintenance of peripheral allograft tolerance (58, 59). Since these cells can be isolated from the spleens of tolerant mice, their presence can be evaluated in co-culture MLR assays (54-56). However, our in vitro results did not demonstrate a suppressive effect on anti-donor responses when naïve lymphocytes were co-cultured with lymphocytes from tolerant mice. This suggested that regulatory T cells are not present in the spleens of long-term engrafted mice treated with anti-ICOS and anti-CD40L. However, the absence of suppression in vitro may be due to a functional and not a physical lack of regulatory T cells, since their effector responses may be specific for islet alloantigen only. The generation of regulatory T cells during the tolerizing regimen occurs in the presence of donor islet antigen and not donor hematopoeitic cells. As a result, the specificity of these cells may be restricted to islet antigen alone, rendering them functionally undetectable in vitro. Another possible explanation, which is not mutually exclusive to the first, is that these cells are limited in their location to the allograft and are therefore absent in the spleen. Recently, Graca et al. (57) demonstrated that CD4$^+$ regulatory T cells were present in tolerated skin allografts and were capable of mediating dominant transplantation tolerance. Immunohistology of tolerated islet allografts from mice treated with anti-ICOS and anti-CD40L revealed marked peri-islet staining for CD4$^+$ and CD25$^+$ T cells, suggesting the presence of an intra-graft population of regulatory T cells. Sakaguchi's group recently reported on a novel transcription factor specifically expressed by CD4$^+$CD25$^+$ regulatory T cells named *Foxp3* (60). They demonstrated that retroviral transfer of *Foxp3* could convert naïve T cells into a regulatory phenotype, indicating that *Foxp3* is a key gene for the development of regulatory T cells. This gene represents a specific marker for regulatory T cells, unlike CD25, CD45RB, and GITR, which are also expressed on activated, effector or memory T cells. Using a novel immunoperoxidase stain, we demonstrated that *Foxp3* was expressed in tolerated islet allografts from mice treated with anti-ICOS and anti-CD40L, suggesting that a proportion of $CD4^+CD25^+$ T cells are specifically of a regulatory phenotype.

As an in vivo functional test for dominant regulatory tolerance, islet allografts bearing intra-graft T cells from tolerant mice were re-transplanted into streptozotocin-treated naïve B6 mice, and followed for rejection. Despite the presence of an intact alloreactive T cell repertoire in the naïve recipients, none of the re-transplanted grafts were rejected beyond 100 days. In order to confirm that long-term acceptance of these grafts was due to an intra-graft dominant regulatory mechanism, and not as a consequence of reduced immunogenicity of the graft secondary to being healed-in (53) and/or depleted of passenger leukocytes (87), we re-transplanted islet allografts from control B6-RAG-KO mice, which had also been allowed to heal in place prior to re-transplantation. In contrast to islet allografts from tolerant treated mice, islet grafts from control RAG mice were rejected, confirming that intra-graft T cells can in fact constrain pathogenic alloresponses in vivo. Whether these cells operate in a cell contact dependent manner (88, 89), through the release of cytokines such as IL-10 (59, 90) or TGF-β (91), or both, remains a matter of speculation.

Having demonstrated the efficacy of combined anti-ICOS and anti-CD40L blockade in the regulation of alloimmune responses, we proceeded to test this strategy in the prevention of autoimmune diabetes in the NOD mouse. We found that while temporary monotherapy with either anti-ICOS or anti-CD40L reduced the onset of diabetes, the combination of these agents resulted in a more profound, significant reduction in diabetes to a mere 11%. In our study, therapy was administered after the onset of insulitis, but prior to the onset of diabetes. The timing of costimulation blockade was based on evidence that blockade of ICOS signaling during antigen priming can result in more severe disease, whereas blockade during the efferent immune response, that is, immediately prior to disease onset, can prevent its progression (92). The synergy between ICOS and CD40L in controlling autoimmune responses in this model is not unexpected given that blockade of these molecules have separately been reported to play critical roles in several experimental autoimmune diseases (92-98). Their potent effect in the primary prevention of diabetes warrants further investigation of this strategy in promoting allograft acceptance in NOD recipients.

In summary, we have demonstrated that combination treatment with anti-ICOS and anti-CD40L is a potent strategy in inducing long-term islet allograft acceptance. The maintenance of donor specific tolerance despite the presence of alloractive T cells is dependent of an active regulatory mechanism that involves the presence of regulatory T cells at the site of the tolerated allograft. These cells express CD4, CD25, and *Foxp3* and are capable of mediating a dominant suppressive effect on pathogenic alloimmune responses. In addition, we have shown that this combination therapy can also significantly reduce the onset of primary autoimmune diabetes in NOD mice, indicating that its effectiveness is not limited to controlling alloimmune responses. These findings underscore the efficacy of simultaneous blockade of ICOS and CD40L signaling as a potential therapy in clinical islet transplantation, and emphasize the need for further studies in large animal models.

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CHAPTER SEVEN

STABLE MIXED CHIMERISM ESTABLISHED BY RAPAMYCIN-BASED NONTOXIC CONDITIONING INDUCES TOLERANCE TO ISLET GRAFTS

Bin Luo MD[1], Sulaiman A. Nanji MD[1], Colleen D. Schur BSc[1],
Rena L. Pawlick BSc[1], Colin C. Anderson PhD[1], A.M. James Shapiro MD, PhD[1]

[1] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada

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7.1 INTRODUCTION

The establishment of stable mixed chimerism through bone marrow transplantation is widely acknowledged as a reliable and robust method of tolerance induction. However, concerns regarding toxicities associated with recipient preconditioning, as well as the threat of graft-versus-host disease (GVHD), have precluded clinical application of this approach. Recently, several nonmyeloablative conditioning strategies, based on costimulation blockade, with or without conventional immunosuppresive drugs, have been developed to induce chimerism and donor specific tolerance in mouse models (1-6). With mega dose bone marrow infusion and costimulation blockade, low level chimerism and indefinite skin allograft acceptance can be achieved in fully MHC mismatched (7) or complete mismatched combinations, even without any cytoreductive treatment (8). However, thromboembolic complications with select costimulation blockade strategies in nonhuman primates (9) and in clinical trials (10), as well as the unavailability of mega dose bone marrow cells, limit the clinical application of these attractive protocols.

Rapamycin, a potent inhibitor of T cell proliferation, and an important immunosuppressive component in the "Edmonton Protocol" for clinical islet transplantation (11), has been shown to promote allogeneic bone marrow engraftment and prevent GVHD under sublethal irradiation (12, 13). Hale et al. have demonstrated that a single injection of high dose rapamycin, after host lymphocyte depletion and before mega dose BMT, promoted induction of mixed chimerism and donor specific skin tolerance in a fully MHC-mismatched combination (14). Wu et al. found that rapamycin montherapy, combined with donor specific transfusion prior to BMT, was as effective as costimulation blockade under an irradiation-free conditioning therapy with busulfan and cyclophosphamide (6). The purpose of this study was to determine if our nontoxic protocol, which does not require costimulation blockade, high dose irradiation or mega dose bone marrow, could lead to establishment of mixed chimerism and tolerance to donor islet grafts.

7.2 MATERIALS AND METHODS

7.2.1 ANIMALS

C57BL/6 (H-2^b), BALB/c (H-2^d) and CBA/J (H-2^k) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under standard conditions and had access to food and water ad libitum. All experiments were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

7.2.2 BONE MARROW TRANSPLANTATION AND TREATMENT PROTOCOLS

BALB/c bone marrow cells were prepared by flushing the femoral and tibia bones with PBS followed by passing the cell suspensions through a nylon mesh. Viable cells were counted and adjusted to 80×10^6 cells/ml before injection. Eight to 12 week-old male C57BL/6 mice were given low dose total body irradiation (TBI, 1-3 Gy as indicated) on day -1 by using a ^{137}Cs irradiator (Gammercell 40, Atomic Energy of Canada, Ottawa, Ontario) at an exposure rate of approximately 0.56 Gy/min. 40×10^6 unmodified BALB/c bone marrow cells (0.5 ml) were injected intravenously into C57BL/6 recipient mice on day 0. Rapamycin (Rapamune, Wyeth Canada) was diluted to 0.2 mg/ml with normal saline, and injected intraperitoneally (i.p.) at 3mg/kg immediately after BMT, and then daily for a total of 28 days. In a separate cohort of mice, recipient lymphocytes were depleted before BMT by rabbit antimouse lymphocyte serum (ALS, Accurate Chemical & Scientific, NY) at a dose of 0.3 ml i.p. on day -5 and day -2.

7.2.3 FLOW CYTOMETRY

Phenotyping was performed at multiple time points beginning at 4 weeks after BMT. Recipients were bled from the tail and collected blood samples were stained with both donor and recipient specific mAbs. Cells were incubated with PE-conjugated anti-H-2D^d (34-2-12) mAb and biotinylated anti-H-2D^b (KH95) mAb for 15 min at 4°C and then washed. Cell-bound biotinylated mAb was detected with APC-streptavidin. Lymphocytes were gated and the percentage of donor cell in recipients was defined as H-2D^d-PE positive cells (donor) divided by the total number of APC-steptavidin-positive cells (recipient) plus H-2D^d-PE positive cells (donor). For analysis of multilineage chimerism, PE-conjugated anti-H-2D^d mAb, FITC-conjugated TCR (H57-597), Tricolor-conjugated CD19 (6D5), Biotin-conjugated Gr-1 (RB6-8C5) and Tricolor-conjugated CD11b (M1/70.15) mAbs were used to label donor T cells, B cells, granulocytes and macrophages, respectively. For analysis of TCR Vβ families, recipient peripheral blood white blood cells were stained with specific FITC-conjugated anti-Vβ5.1,5.2 (MR9-4), PE-conjugated anti-Vβ8 (F23.1) and Tricolor-conjugated anti-CD4 (CT-CD4) mAbs. 20,000 lymphocytes were gated for Vβ analysis. CD19, CD11b and CD4 mAbs were obtained from Caltag Laboratories (Burlingame, CA), and all other mAbs were purchased from BD Pharmingen (San Diego, CA). Data was analyzed with a FACSCalibur (Becton Dickson, Sunnyvale, CA).

7.2.4 INDUCTION OF DIABETES, ISLET ISOLATION AND TRANSPLANTATION

After BMT and detection of mixed chimerism, C57BL/6 mice were rendered diabetic by a single intravenous injection of streptozotocin (200mg/kg, Sigma-Aldrich, Canada). Onset of diabetes was confirmed with a blood glucose level > 20 mmol/L (Glucometer, Lifescan) for 2 consecutive days. BALB/c islets were isolated as previously described (15,16). Briefly, BALB/c pancreata were first harvested after collagenase (1mg/ml, Sigma-Aldrich, Canada) perfusion via the common bile duct, followed by agitated digestion for 15 minutes at 37° C. After islet purification on discontinuous Ficoll gradients (Sigma-Aldrich, Canada), approximately 400 islets were handpicked and transplanted under the left renal capsule of diabetic recipient mice. To test for donor specific tolerance, mice remaining normoglycemic beyond 100 days were first nephrectomized of their graft-bearing kidneys. Once a return of hyperglycemia was confirmed, these mice were then transplanted with a second same donor (BALB/c) or third party (CBA/J) islet graft under the right renal capsule. Serum glucose levels were monitored and graft rejection was defined as a return to serum glucose levels in excess of 15 mmol/L for 2 successive days.

7.2.5 MLR ASSAY

Spleens cell suspension was prepared with nylon mesh. Various dilutions of responder splenocytes were cultured with 1×10^6 irradiated (15 Gy) stimulator spleen cells in 0.2 ml Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol. After 3 days of culture in a 37°C, 5%CO_2 incubator, 1μCi ^3H thymidine was added to each well. Cells were harvested about 14-18 hours later and thymidine incorporation was measured using a liquid scintillation counter (1450 Microbeta, Wallac, Turca, Finland).

7.2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using a two-tailed Student's *t*-test for comparison of means with unequal variances. A p value of less than 0.05 was considered statistically significant.

7.3 RESULTS

7.3.1 *INDUCTION OF MIXED CHIMERISM WITH 3 GY TBI AND RAPAMYCIN*

Initial studies were aimed to determine if mixed chimerism could be achieved with 3 Gy TBI conditioning, followed by rapamycin monotherapy post-BMT for 4 weeks. It has been reported that 3 Gy TBI and 4-week post BMT (15-20 million) rapamycin monotherapy (0.2 mg/kg) could not establish chimerism (4). We attempted a protocol based on 40 million bone marrow cells for BMT, which is still within the range that can be achieved clinically (2), and a high dose of rapamycin at 3 mg/kg as post-BMT treatment. As shown in Table 7-1, in three repeated experiments, 13/15 recipients developed high levels of chimerism at 8 weeks. However, this level of chimerism gradually declined in some recipients, and was eventually lost in 4 mice at 12 weeks (data not shown), The remaining 9 mice maintained chimerism and good health beyond 20 weeks after BMT. All chimeric mice gained body weight and had no clinical signs of GVHD. One chimeric mouse died with a functional islet graft 21 days after transplantation, 97 days after BMT, and another chimeric mouse died 145 days after BMT. While the cause of death in these mice is unknown, there was no evidence of GVHD, malignancy or chronic infection. When the dose of rapamycin was decreased to 2mg/kg, only transient chimerism was detected within 12 weeks (data not shown), indicating that the dose of rapamycin is a critical factor in establishing chimerism. Moreover, decreasing the dose of TBI also impaired the establishment of chimerism at 8 weeks (data not shown). These results indicate that 3Gy TBI and 3 mg/kg rapamycin is the minimal conditioning needed to achieve mixed chimerism across a fully mismatched barrier.

Treatment	Chimeric mice, donor chimerism (mean ±SD) weeks, post-BMT			
	8		20	
TBI 3Gy	0/5		0/5	
TBI 3Gy / Rapa	13/15	41±19%	9/11[b]	31±18%
ALS / TBI 3Gy / Rapa	9/9	53±17%	9/9	63±19%
ALS / TBI 3Gy	0/5		ND	

Table 7-1: Induction of chimerism with 3 Gy TBI and rapamycin[a]
C57BL/6 mice received TBI (d-1), 40×10^6 BALB/c bone marrow cells (d 0) and rapamycin (3mg/kg, ip, d 0-28) ± ALS (0.3 ml, ip, d-5, -2). Donor lymphocytes were monitored by flow cytometry. (b) 2 nonchimeric mice were sacrificed because of hyperglycemia (rejection of islet graft), 1 chimeric mouse died on d 145 post-BMT, and 1 chimeric mouse died on d 21 post islet transplantation with a functional graft (d 97 post-BMT), cause unknown.

Furthermore, the toxicity of the conditioning regimen was evaluated by monitoring the peripheral WBCs in recipient mice treated with 3 Gy TBI (day -1), 40 million BALB/c bone marrow cell infusion and rapamycin (3 mg/kg, day 0-28) (Figure 7-1). The WBC counts dropped to a nadir at day 7 (3.3×10^3/mm^3) and recovered to the normal range by 4-6 weeks. Moreover, there was no mortality or morbidity in control mice treated with 3 Gy TBI/rapamycin in the absence of bone marrow infusion.

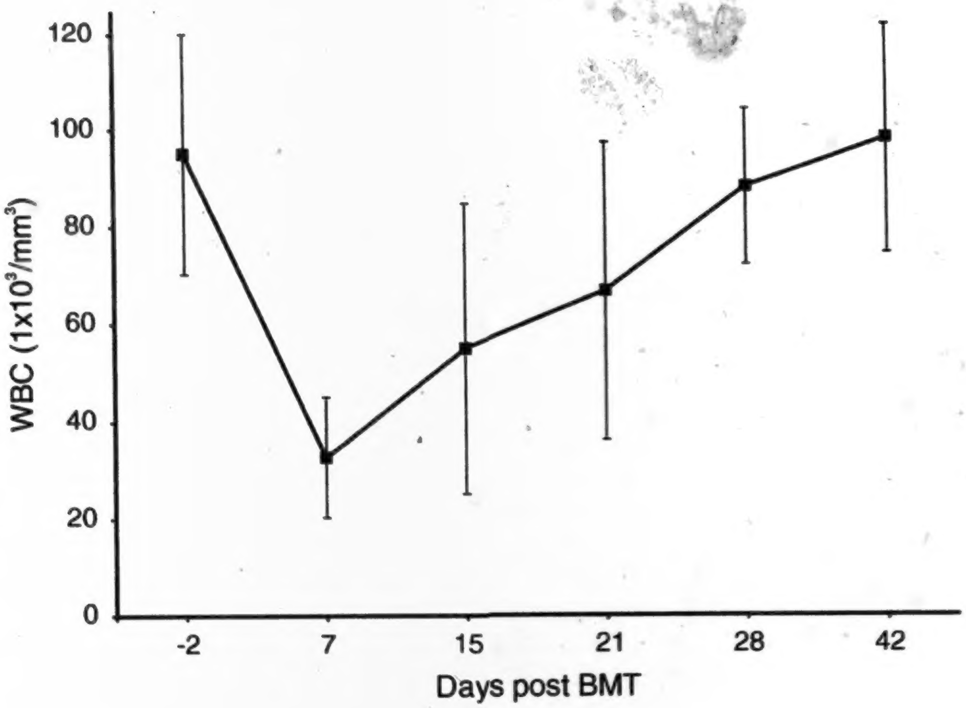

Figure 7-1: Toxicity of conditioning therapy.
Effect of TBI 3 Gy (d 0), BMT (BALB/c, 40 million) and rapamycin (3 mg/kg, d 0-28) on recipient WBCs over time (n=5). Error bars represent standard deviation.

7.3.2 LYMPHOCYTE DEPLETION IN COMBINATION WITH 3GY TBI AND RAPAMYCIN INDUCES STABLE MULTILINEAGE CHIMERISM

Although TBI 3Gy and rapamycin treatment was effective at inducing mixed chimerism, the level of chimerism achieved could not be maintained in all mice. One possibility for the loss of chimerism may be the rejection of donor hematopoeitic cells by pre-existing host mature T cell that escaped the nonmyeloablative conditioning. We hypothesized that depletion of host lymphocytes prior to BMT would overcome the alloresistance to donor bone marrow engraftment and lead to stable mixed chimerism. This was tested by injection of ALS (0.3 ml IP) on day -5 and day -2. As shown in Table

7-1, in two individual experiments, all mice treated with ALS /TBI 3Gy/ rapamycin developed a high level of mixed chimerism at 8 weeks, which remained stable long term. The importance of post-BMT rapamycin treatment was again demonstrated, as chimerism could not be detected under ALS and TBI 3Gy treatment alone (Table 7-1).

It has previously been shown that donor T cells could not be detected in chimeras induced by ALS, rapamycin and mega-dose bone marrow infusion (14). We evaluated multilineage donor engraftment in mice treated with TBI 3Gy/rapamycin with or without ALS. As depicted in Table 7-2, engraftment was clearly detected in the T cell, B cell, macrophage and granulocyte lineages. Moreover, evaluation of donor engraftment in the marrow, spleen, and thymus in recipient mice at 40 weeks revealed that multiorgan engraftment was achieved (Table 7-3). Most importantly, donor cells could be detected in recipient thymuses.

Treatment	Donor chimerism, mean ±SD			
	T cells	B cells	Macrophage	Granulocytes
TBI 3Gy/Rapa (n=4)	24± 11%	62 ±12%	49± 7%	57± 7%
ALS/TBI 3Gy/Rapa (n=5)	53± 29%	64± 20%	89± 3%	91± 3%

Table 7-2: Multilineage chimerism in mice treated with rapamycin-based protocol. C57BL/6 mice were treated with TBI (d-1), rapamycin (3mg/kg, d 0-28) and 40×10^6 BALB/c bone marrow cells (d 0) ± ALS (0.3 ml, d-5 and –2). Multilineage chimerism in peripheral blood was detected by flow cytometry 20 weeks after BMT.

Treatment	donor chimerism			
	Blood	Spleen	Bone marrow	Thymus
TBI3Gy/Rapa	49%	43%	39%	15%
TBI3Gy/Rapa	24%	33%	16%	18%
ALS/TBI3Gy/Rapa	50%	53%	49%	45%
ALS/TBI 3Gy/Rapa	82%	81%	70%	71%

Table 7-3: Chimerism in multiorgans.
C57BL/6 chimeric mice were sacrificed at 40 weeks after BMT and the percentage of donor lymphocytes in different organs was detected by flow cytometry.

7.3.3 DEPLETION OF RECIPIENT LYMPHOCYTES WITH ALS FACILITATED THE INDUCTION OF STABLE CHIMERISM WITH REDUCED TBI OR IRRADIATION-FREE CONDITIONING

In order for chimerism strategies to be more desirable in the clinic, a reduction, or ideally the elimination, of irradiation in the conditioning regimen is critically important. In view of the robust chimerism induced by the ALS /TBI 3Gy /rapamycin regimen, we were interested to determine if chimerism could still be established despite a reduction in TBI. As shown in Table 7-4, in two separate experiments, a reduction to 2 Gy or 1 Gy TBI conditioning resulted in the induction of stable mixed chimerism in 9 of 10 mice. Although the level of chimerism in mice treated with ALS /TBI 1Gy/ rapamycin was significantly lower than that of mice treated with either TBI 3Gy or TBI 2Gy ($p < 0.01$), the level of chimerism in these mice was maintained long-term. Complete elimination of TBI resulted in a failure to induce chimerism with ALS and rapamycin treatment alone.

In an attempt to create an irradiation-free conditioning regimen, we replaced 1Gy TBI with cyclophosphamide (CTX, 200mg/kg day –1, i.p.) and found that this regimen was also effective at inducing long-term stable chimerism in all treated mice (Table 7-4). In summary, therefore, these results indicate that rapamycin monotherapy post BMT allows for long-term stable chimerism in a low dose TBI conditioning protocol. Additionally, the dose of TBI can be further decreased to 1 Gy or replaced by CTX by pre-BMT lymphocyte depletion.

Treatment	Chimeric mice, donor chimerism (mean ±SD) weeks, post-BMT			
	8		20	
ALS / TBI 2Gy / Rapa	9/10	47±21%	9/10	67±13%
ALS / TBI 1Gy / Rapa	9/10	15±7%	9/10	21±9%
ALS / Rapa	0/4		ND	
ALS / CTX / Rapa	4/4	12±4%	4/4	12±3%

Table 7-4: Induction of chimerism with low dose TBI or Cyclophosphamide conditioning and rapamycin treatment.
C57BL/c mice received TBI (d-1) or CTX (200 mg/kg, d-1), BALB/c bone marrow cells (d 0) and rapamycin (3mg/kg, ip, d 0- 28) combined with ALS (0.3 ml, ip, d-5,-2). Donor lymphocyte chimerism was monitored.

7.3.4 DONOR REACTIVE T CELLS ARE DELETED IN CHIMERIC MICE

BALB/c mice express I-E, which is required to present superantigens that facilitate the deletion of Vβ5- and Vβ11-bearing T cells in the thymus. Unlike BALB/c mice, B57BL/6 mice do not express I-E and, therefore, bear 2-3% $CD4^+V\beta5^+$ T cells and 4-5% $CD4^+V\beta11^+$ T cells. The absence of these T cells in B6 mice rendered chimeric by BALB/c BMT serves as an indicator of central clonal deletion of anti-donor T cells (1, 18). We tested chimeric mice for the presence of $V\beta5^+$ T cells and used Vβ8-bearing T cells as a control since these cells are not deleted in either mouse strain. As shown in Figure 7-2, chimeric mice treated with ALS /TBI 3Gy/ rapamycin had significantly lower levels of $CD4^+V\beta5^+$ T cells at 20 weeks after BMT, as compared to naïve C57BL/6 mice ($p<0.001$). Moreover, as expected, $CD4^+V\beta5^+$ T cells were not deleted in nonchimeric mice treated with TBI 3Gy and BMT, as evidenced by comparable levels with naïve C56BL/6 mice. Interestingly, $CD4^+V\beta8^+$ T cells did not reduce but increased significantly in chimeric mice ($p<0.001$). Although this trend has also been reported elsewhere (19), its significance, if any, remains unclear at this time. These results, together with evidence of donor cell engraftment in the host thymus (Table 7-3), indicate that chimeric mice exhibit donor-specific central T cell deletion.

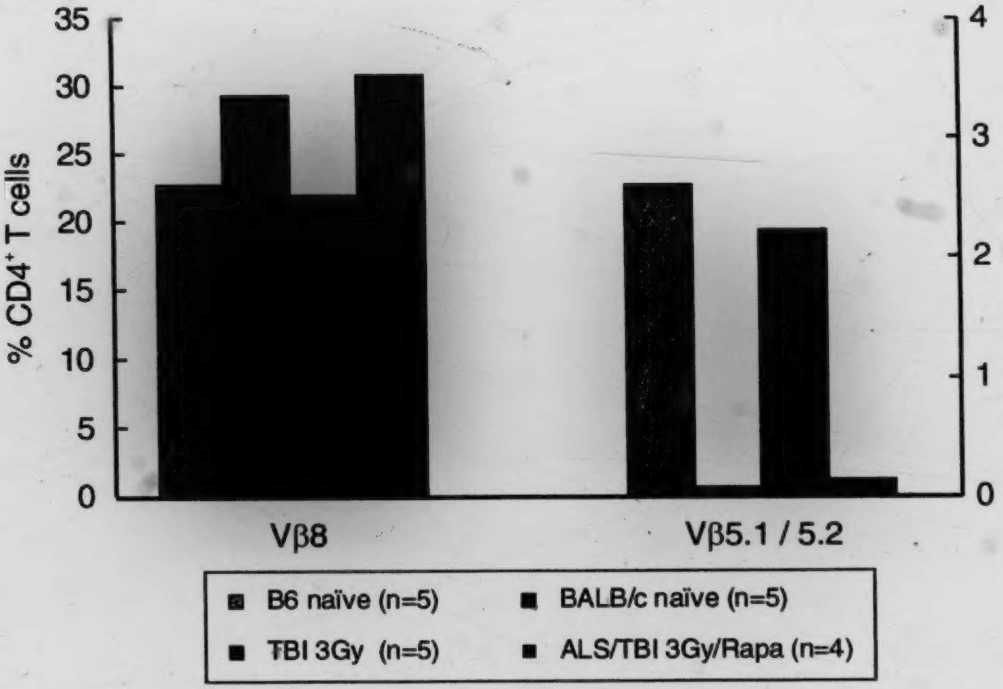

Figure 7-2: CD4$^+$/Vβ5$^+$ donor reactive T cell depletion in chimeric mice.
Mice treated with ALS /TBI 3Gy/ Rapamycin developed chimerism and specifically deleted CD4$^+$/Vβ5$^+$ T cells, while recipients of TBI alone were nonchimeric and maintained CD4$^+$/Vβ5$^+$ T cells. Vβ family detection was performed at 20 weeks post-BMT.

7.3.5 DONOR SPECIFIC ISLETS ALLOGRAFTS ARE ACCEPTED LONG-TERM IN CHIMERIC MICE

Having established a regimen that induces stable mixed chimerism and central deletion of alloreactive T cells, we proceed to test for acceptance of donor-specific islet allografts in chimeric mice. At least 4 weeks after rapamycin treatment (8 weeks after BMT), chemical diabetes was induced in chimeric recipients, followed by islet transplantation. As shown in Table 7-5, all chimeras treated with TBI 3Gy/rapamycin (n=6), ALS /TBI 2Gy/ rapamycin (n=5) or ALS /TBI 1Gy/ rapamycin (n=5) accepted donor islet grafts long-term (>100 day). These mice subsequently underwent nephrectomy of their graft-bearing kidneys and demonstrated a return to hyperglycemia, thereby confirming the long-term functionality of the islet allograft. These mice were subsequently re-challenged with a second same donor or third party (CBA/J) islet graft. All chimeric mice accepted second same donor grafts long-term, and rapidly rejected third party islet grafts, indicating the presence of donor-specific tolerance in vivo.

Treatment	BMT	Chimerism	n	First donor (BALB/c) islet graft survival (d)	Regrafts survival (d)	
					Donor (BALB/c)	Third party (CBA/J)
TBI 3Gy	+	–	5	7, 8×3, 9	ND	
ALS/TBI 3Gy	+	–	4	13, 14, 15, 16	ND	
TBI 3Gy/Rapa	–	–	4	20, 21, >100, 129[b]	>70	ND
TBI 3Gy/Rapa ALS/TBI 2Gy/Rapa ALS/TBI 1Gy/Rapa	+	+	16	>100× 16	>50×4, >100× 4	11,13,15,17

Table 7-5: Donor specific tolerance to islet allografts in chimeric mice
C57BL/6 mice from different conditioning regimens underwent chemical induction of diabetes, followed by implantation of a BALB/c islet graft. (b) The glucose level of this mouse increased to 10 μmol/l at approximately day 90. Nephrectomy and a second islet transplant was not performed in this mouse

All mice treated with TBI 3Gy or ALS/TBI 3Gy without rapamycin did not develop chimerism and rapidly rejected their primary BALB/c islet graft. Of the four mice treated with TBI 3Gy/rapamycin alone without bone marrow infusion, two rejected their BALB/c islet graft at days 20 and 21, one rejected at day 129 (although blood glucose remained elevated beyond 10 mmol/l from day 90), and one demonstrated long-term acceptance of the first graft and acceptance of the second same donor graft >70days (Table 7-5). These results indicate that although TBI 3Gy/rapamycin treatment showed some nonspecific immune suppressive effects, only chimeric mice established with the rapamycin-based nontoxic conditioning regimen demonstrated donor specific tolerance to islet allografts.

7.3.6 MICE DEMONSTRATING STABLE MIXED CHIMERISM FOLLOWING THE RAPAMYCIN-BASED NONTOXIC PROTOCOL DEMONSTRATE DONOR SPECIFIC UNRESPONSIVENESS IN MLRS

Four months after BMT, in vitro proliferation of lymphocytes from chimeric and naïve C57BL/6 mice were tested in response to recipient strain (C56BL/6), donor strain (BALB/c) and third-party (CBA/J) stimulators (Figure 7-3). Lymphocytes from chimeras remained fully responsive to third-party stimulators, but were unresponsive to donor type BALB/c and recipient type C57BL/6 stimulators, indicating that chimeric mice had established donor specific tolerance.

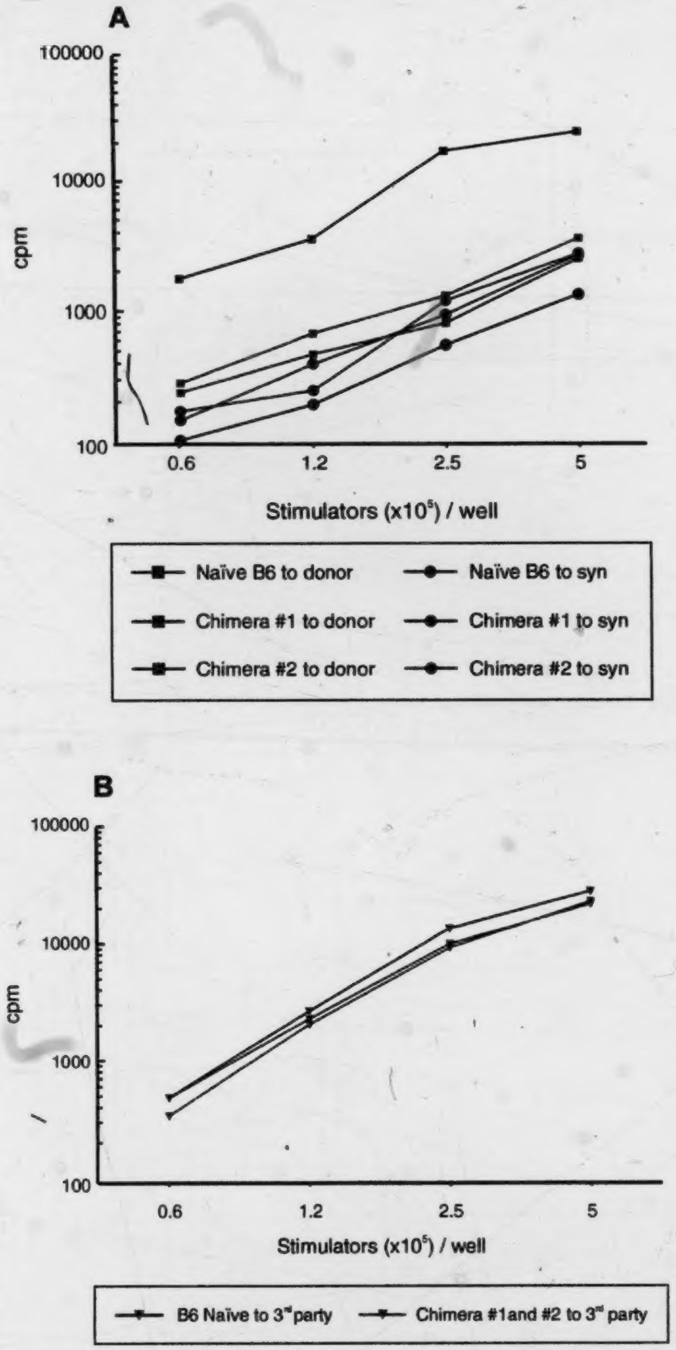

Figure 7-3: Chimeras demonstrate donor specific tolerance in vitro. Lymphocytes from chimeras were hyporesponsive to donor type (BALB/c) and recipient type (C57BL/6) stimulators (A), but remained fully responsive to third-party (CBA/J) stimulators (B)

7.4 DISCUSSION

The induction of mixed chimerism is a promising approach to induce transplantation tolerance. The development of nontoxic, clinically applicable strategies to induce stable mixed chimerism is an important research goal. Recently, Millan et al reported that mixed chimerism could be achieved in patients of combined kidney and hematopoietic progenitor transplants conditioned with total-lymphoid irradiation and antithymocyte globulin, followed by withdrawal of steroids and cyclosporine (20).

In the present study, we tested the efficacy of rapamycin as a single immunosuppressive drug in facilitating the induction of chimerism after low dose TBI conditioning and BMT. We found that mixed chimerism could be established across a complete mismatched barrier with 3Gy TBI and post-BMT rapamycin monotherapy. Rapamycin is a macrolide antibiotic with a similar biochemical structure to cyclosporine A and FK506 (21). However, unlike cyclosporine A and FK506, rapamycin inhibits the ability of lymphocytes to proliferate in response to IL-2 but does not affect costimulation-dependent IL-2 production (22). It is reported that full T cell activation (signal 1 plus 2) in the presence of rapamycin results in profound T cell anergy (23), suggesting a possible mechanism by which rapamycin is effective in our costimulation blockade-free protocol. We found that a dose of rapamycin at 3 mg/kg for the duration of 28 days is the minimal requirement for the induction of chimerism. Although this dose is approximately 10 fold higher than that typically effective in rodent transplantation studies, it appeared to be safe, since all mice in our study tolerated the 4-week treatment without any evidence of ill health. However, this elevated dose of rapamycin was found to inhibit lymphocyte recovery, since fewer lymphocytes were detected in the peripheral blood at the termination of rapamycin therapy (4 weeks), as compared to mice treated with TBI 3Gy alone. Nevertheless, lymphocytes recovered to within the normal range by 6-8 weeks.

Moreover, of approximately 200 chimeric mice treated with our rapamycin-based protocol, no clinical signs of GVHD were ever evident and only one mouse was found to have an ulcerative skin lesion on its back (at 60 weeks after BMT). This was unlike the rapamyicn-induced skin lesions often reported on the limbs of bone marrow recipients undergoing high dose irradiation (8-9 Gy) and rapamycin treatment (24). Finally, several of our chimeric mice have been kept for more than 1 year, and still remain healthy.

Although this regimen appears safe, not all the mice treated with TBI 3Gy/rapamycin maintained chimerism indefinitely. However, we found that depletion of lymphocytes with ALS prior to BMT led to higher levels of chimerism that remained stable long-term. The importance of rapamycin was also confirmed in this protocol since ALS/TBI 3Gy treatment alone could not induce chimerism. Furthermore, we found that the dose of TBI in this regimen could be reduced to 1 Gy without compromising the establishment of chimerism. While 1 Gy is a reasonable dose for clinical application, complete elimination of irradiation would be preferable. Therefore, we replaced TBI with CTX, and found that stable chimersim could still be reliably achieved.

In this protocol, rapamycin may have effects beyond that of immunosuppression. For instance, in a rat cardiac allograft model, rapamycin treatment at 1mg/kg for 14 days caused thymic atrophy with accelerated apoptosis of $CD4^+CD8^+$ thymocytes (25). Therefore, rapamycin may play a role to inhibit T cell maturation in the thymus. Colson et al. demonstrated 3Gy TBI and antilymphocyte globulin could also significantly eliminate the $CD4^+CD8^+$ T cell in the thymus, however, this immature population completely recovered in one week (26). Our post-BMT rapamycin therapy may delay the maturation of new T cells, allowing these immature T cells a chance to encounter the newly engrafted donor cells in the thymus. This may explain why the combination of ALS, TBI and rapamycin can induce stable chimerism.

We also evaluated the strength of tolerance to islet grafts in mice undergoing this protocol and found that chimeric mice accept donor strain islet grafts indefinitely, whereas nonchimeirc control mice rapidly reject these grafts. Long-term engrafted mice were subsequently re-challenged and demonstrated acceptance of second donor grafts and rejection of the third-party grafts, indicating that donor specific tolerance was established (Table 7-5). However, the possibility that islet allograft acceptance may in part be attributable to the weak immunosuppressive properties of streptozotocin, or to the diabetes it induces, cannot be excluded at this time (27).

Similar to other successful chimerism protocols, central clonal deletion was found to be the main mechanism for the maintenance of tolerance, as evidenced by donor hematopoietic cell engraftment in the host thymus (Table 7-3) and deletion of superantigen-specific $CD4^{+}V\beta5^{+}$ T cells in chimeric mice (Figure 7-2). Hale et al demonstrated that chimerism could be established in mice treated with ALS and a single injection of high dose rapamycin, followed by mega-dose bone marrow infusion without any irradiation (14). Despite a lack of donor T cells, tolerance to skin allografts could still be achieved (14). In their protocol, rapamycin was a critical component. Although ALS was not myelosuppressive, in the absence of irradiation, the single high dose of rapamycin (24 mg/kg) given before BMT might have had such effects. Others have shown that high concentrations of rapamycin can inhibit bone marrow cell growth in vitro (28). In a congenic combination (C57BL/6 to B6Ly5.2), we found that stable multilineage chimerism could be achieved with a single injection of rapamycin at 100 mg/kg one day before BMT (unpublished data).

Recently, Li et al described a nonlethal and costimulation blockade-free protocol, consisting of ALS depletion (day -3), 1 Gy TBI (day 0) and a single dose of CTX (day +2) as conditioning therapy, and two infusions of 30 million bone marrow cells (day 0 and 3) (29). Stable mixed chimerism and donor specific tolerance to islet grafts could be achieved with this protocol. CTX in this combination was shown to delay the reconstitution of $CD4^{+}CD8^{+}$ in the thymus (26). This protocol is similar to ours, and it can be argued that one injection of CTX post-BMT is simpler than 4-weeks of rapamycin treatment. However, an advantage of our rapamycin-based approach is that TBI can be completely eliminated and only one BMT is needed.

In summary, we have demonstrated that mixed chimerism can be induced with low dose irradiation and post-BMT rapamycin treatment. The addition of ALS given prior to BMT resulted in higher levels of chimerism that could be maintained indefinitely. The establishment of chimerism with this approach also induced donor specific tolerance to islet grafts. The simplicity of this protocol, the acceptable safety profile, and the robustness of tolerance induced, indicates that this protocol has potential to be tested further in large animal models.

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CHAPTER EIGHT

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 DISSERTATION FINDINGS AND IMPLICATIONS

Since the first report of experimental tolerance induction more than half a century ago, the development of clinical tolerance protocols remains a highly sought-after but elusive goal. The expanding repertoire of progressively more potent and less toxic immunosuppressive agents has generated considerable enthusiasm for therapies that may facilitate the induction of tolerance. T cell signaling molecules are exciting immunomodulatory targets, and the development of agents designed to specifically block these molecules is one of most promising emerging therapies.

Several new costimulatory pathways have been characterized in recent years, and additional signaling molecules continue to be identified. These discoveries have led to an appreciation that many molecules act to influence immune responses and that overcoming allograft rejection in the clinic will likely require the combination of agents that act synergistically to contend with the inherent redundancy of the immune system. Presently, any strategy to induce tolerance involves the use of immunosuppressive agents for a limited period of time until a stable tolerant state has been achieved. Therefore, identifying those agents that lend themselves to tolerance induction is of paramount importance in developing novel protocols for potential clinical application.

With these concepts in mind, the primary focus of this thesis was to develop strategies to induce tolerance in rodent models of islet transplantation, with potential for future clinical translation. The blockade of costimulation and the establishment of mixed chimerism were studied in these models using existing tolerance-promoting immunosuppression such as rapamycin, as well as an experimental blocking antibody to a novel costimulatory pathway, ICOS:B7RP-1. The functions of this pathway and its potential role in islet allograft rejection and tolerance have not been previously investigated. Characterizing the influence of ICOS on alloimmune responses, the potential mechanisms of action, and the effect of ICOS blockade either as monotherapy or in combination with existing promising agents, were primary aims of this work.

An interesting concept in the study of transplant immunology is the tenet that the rate of allograft acceptance, and hence the response of an allograft to tolerance protocols, is organ- and tissue-dependent, with more stringent allografts such as skin and islets being more susceptible to rejection. While several intrinsic factors such as the vascularity of the allograft, the graft size and the presence of tissue specific antigens have all been proposed to influence the alloimmune response, we demonstrated that factors extrinsic to the tissue also contribute to the hierarchy in allograft acceptance. In examining the differences between cardiac and islet allograft acceptance, we identified the presence of secondary lymphoid tissue as a critical factor in increasing the susceptibility of islet allografts to rejection, particularly under conditions of ICOS costimulation blockade. This suggested that the presence of intact lymphatic drainage renders islet allografts more resistant to costimulation blockade for tolerance induction. Therefore, agents that alter lymphocyte trafficking such as specific anti-chemokine mAbs or FTY720, may prove to be synergistic with anti-ICOS mAb or other costimulation blockade-based therapies and warrant further investigation.

In a number of transplant models, the efficacy of an individual agent can be augmented by combination with other agents. In this regard, while treatment with anti-ICOS mAb alone was ineffective at prolonging islet allograft survival, the combined blockade of ICOS and either CD40L or CD28 signaling proved particularly potent. Moreover, the combination of anti-ICOS mAb and rapamycin also demonstrated synergy in facilitating a state of operational tolerance. When considering any novel therapy for potential clinical translation, one of the critical challenges is the integration of the new approach into currently accepted protocols. Rapamycin is a key immunosuppressant in the Edmonton Protocol and has been shown to synergize with costimulation blockade in facilitating tolerance induction, therefore, the study of anti-ICOS mAb treatment with rapamycin in islet transplantation has potential clinical relevance. Further evaluation of this strategy revealed that it had a potent effect on acute anti-donor proliferative and effector T cell responses in vivo, allowing for the establishment of operational tolerance. While allo-specific responses were not completely eliminated long-term, this strategy was effective in blunting host immune responses in such a manner that indefinite graft acceptance could be achieved with only a limited period of therapy.

The mechanisms through which costimulation blockade facilitate allograft acceptance have been greatly debated. Some maintain that tolerance is due to a loss of function through either deletion or anergy, whereas others claim that tolerance is due to a gain of function through the generation of regulatory T cells. What is becoming more apparent in several models is that both mechanisms are ultimately at work. In costimulation blockade-based strategies, the inhibition of costimulatory signals should ensure that the alloimmune response is controlled in the short-term such that regulatory mechanisms can emerge to a level that can help to maintain tolerance in the long-term. Evaluation of mechanisms with combined blockade of ICOS and CD40L signaling appear to be consistent with this view, where combination therapy impaired alloreactivity sufficiently in order to allow for regulation to emerge as dominant. This regulatory mechanism was found to be operational at the level of the allograft, and was not detected when analyzed in vitro. The discrepancy between the in vivo and in vitro findings suggest that the conditions in the in vitro assays may not adequately resemble the micro-environment that exists in the tolerant recipient, either within the allograft or in the periphery. Therefore, the identification of specific markers to identify regulatory T cells may allow for a more reliable means of evaluating the presence of regulatory mechanisms. One such marker has been recently identified and termed Foxp3. This transcription factor is a key gene in the development of regulatory T cells and serves as a specific molecular marker to identify the presence of these cells. Immunoperoxidase staining for Foxp3 on tolerated islet allografts from mice treated with anti-ICOS and anti-CD40L mAbs revealed increased peri-islet expression, suggesting the presence of intra-graft regulatory T cells. Presently, Foxp3 expression provides an opportunity to uniquely identify regulatory T cells, however, the identification of this critical transcription factor may also serve as a powerful means of transforming naïve T cells into regulatory T cells by Foxp3 transduction. Further studies in this area may lead to a powerful, novel therapeutic approach to transplantation tolerance. Parenthetically, regulatory mechanisms are of particular interest in islet transplantation in view of the fact that linked suppression may facilitate tolerance to the multiple donor pancreata that are sometimes required for one patient to achieve insulin independence. Furthermore, the use of immunosuppressive drugs that may be used in conjunction with agents that promote regulatory tolerance should not interfere with establishment of regulation, as has been speculated with the use of calcineurin inhibitors.

For any tolerance protocol to be successful in islet transplantation, it must effectively contend with both alloimmune and autoimmune processes of graft rejection. Therefore, while the combination of ICOS and CD40L blockade was shown to significantly prevent the onset of primary autoimmune diabetes, it remains to be determined if this strategy is effective in the prevention of both autoimmunity and alloimmunity in a model of allogeneic islet transplantation in the NOD mouse. In addition, the mechanisms associated with the reduction in the onset of spontaneous diabetes require further elucidation and may prove beneficial in other autoimmune models. Moreover, while ICOS blockade appears to be effective at decreasing ongoing pathogenic autoimmune responses in diabetes, it may also play a role in re-establishing tolerance to the affected islet cells. Therefore, treatment with anti-ICOS mAb at the time of diabetes onset may prove to be a useful therapy in restoring normoglycemia.

Taken together, the series of studies in this thesis have provided novel observations on the ICOS:B7RP-1 pathway and have increased our understanding of the mechanisms of how ICOS regulates alloimmune responses. These findings suggest that blockade of ICOS costimulation may have potential therapeutic benefit in islet transplantation, and therefore, may impact on the development of future immunotherapeutic strategies for inducing transplantation tolerance. The effect of ICOS blockade in the autoimmune NOD model further suggests that it may also have a therapeutic role in other immune–mediated diseases besides alloimmune rejection. These results require further confirmation in large animal models, with a view to potential clinical application.

The ICOS mAb used in this work is a rat anti-mouse antibody, therefore, the use of this reagent in large animal models may have negligible therapeutic effect due to the formation anti-rat antibodies. In order to move forward in pre-clinical testing, a more "humanlike" mAb with high binding affinity but less immunogenicity requires development. Using recombinant technology these so called humanized monoclonal products can be constructed such that only the antigen-binding regions are derived from a mouse, while the remainder of the variable and constant regions are of human origin. The human portions of the antibody render it invisible to the immune system, thereby eliminating the antibody response while still maintaining strong binding affinity. However, since the binding region of humanized mAbs remains of foreign origin, it is possible that an anti-idiotype antibody response could develop, but the impact of such a response may be inconsequential. Moreover, since ICOS plays an important role in B cell responses and immunoglobulin class switching, treatment with an anti-ICOS mAb may in of itself block the development of an antibody response. Therefore, the concern of a potential neutralizing antibody response to the reagent may not have any practical significance and may only be a theoretical concern. Finally, another alternative to humanized monoclonal products is creating a human monoclonal antibody, which is entirely of human structure. Currently, the most effective means of producing such a reagent is through transgenic mice that are engineered to produce purely human immunoglobulin molecules. Human mAbs can also be produced from human hybridomas or human B-lymphocyte cell lines immortalized by Epstein-Barr virus; however, these cells lines are unstable and produce only small amounts of mAbs. The production of either humanized or human monoclonal products is a difficult and extremely expensive endeavour. However, once completed, large quantities of the product can be generated for widespread pre-clinical application. Fortunately, a humanized anti-ICOS mAb has already been produced and efforts are currently underway to obtain this agent for nonhuman primate studies.

Studies in larger animal models are critical towards clinical translation since it is clear that what applies to rodents is not often applicable to humans. While many reports claim to induce donor-specific tolerance in rodent models with graft survival beyond 100 days, it is impossible to determine how this standard criteria in rodents translates in terms of longevity for human transplants. While the transplant literature is replete with strategies to induce tolerance in rodents, very few approaches have proven successful in non-human primate models. Of these, the blockade of costimulation has proven promising with reports of long-term kidney and islet allograft survival in non-human primates. Indeed, this approach has also shown success in patients in the treatment of autoimmune psoriasis and in recipients of bone marrow transplants. Despite concerns that outbred species like humans and non-human primates have an enormous immunological history that may prove to be a potent barrier to tolerance, robust tolerance may still be achievable through the combination of agents that synergize with costimulation blockade. For instance, the reduction of the central and peripheral alloreactive T cell repertoire through adjunctive therapies with depletional agents such as anti-CD3 or campath-1H, may prove to play a significant role in facilitating tolerance. However, requirements in the magnitude of deletion and issues of safety with respect to minimizing risks of malignancy and infection in the absence of lymphocytes require further evaluation. Moreover, whether co-administration of donor antigen, in the form of donor blood transfusions or bone marrow infusions, enhances tolerance through T cell depletion and costimulation blockade remain additional challenges to address.

It has been reported that non-human primates that have accepted their islet allografts long-term through anti-CD40L treatment require periodic re-treatment with the mAb to prevent rejection. This implies that maintenance of normal allograft function in patients treated with costimulation blockade may depend on ensuring that adequate levels of the anti-costimulation agent are present at all times to prevent the activation of newly developed alloreactive T cells. Even if this were the case, periodic dosing with specific mAbs in exchange for daily non-specific immunosuppression would be a remarkable development towards achieving tolerance.

With the ongoing identification of novel costimulatory and coinhibitory pathways, the functions of these pathways and their potential interactions in allograft rejection and tolerance require extensive investigation to identify the most promising targets. In view of the complex interactions between these pathways, together with their wide distribution, the safety and efficacy of targeting these pathways in vivo will require meticulous attention as these therapies are translated to primates and humans.

While the blockade of costimulation offers tremendous clinical potential, the induction of mixed allogeneic chimerism has reliably proven to be the most robust approach to tolerance, and arguably represents the strategy of choice for clinical tolerance induction. However, a major challenge is to develop clinically applicable, non-myeloablative conditioning regimens that facilitate bone marrow transplantation and induction of stable chimerism in HLA-mismatched recipients. To this end, we developed a protocol for long-term stable chimerism that relies on rapamycin monotherapy post-BMT in a low dose total body irradiation (TBI) conditioning protocol. Additionally, we found that the dose of TBI could be further decreased or replaced by cyclophosphamide by pre-BMT lymphocyte depletion with anti-lymphocyte serum. This simple protocol proved to be safe in a rodent model and led to donor-specific tolerance to islet allografts through a central deletional mechanism.

While the work in this thesis has outlined two promising approaches to tolerance induction, the question as to which of the two, peripheral mechanisms of tolerance through costimulation blockade or central mechanisms through mixed chimerism, has the qualities of a potential clinical strategy becomes an important question. There are several enticing features of the mixed chimerism approach. First, by functioning through a central deletional mechanism, this strategy generates a robust form of tolerance that has consistently demonstrated endurance to the most rigorous tests of transplantation tolerance. Conversely, peripheral deletional mechanisms do not necessarily maintain complete elimination of the donor-reactive T cell repertoire, therefore, tolerance may be overcome under certain conditions allowing for existing donor-reactive T cells to mediate pathogenic immune responses. To increase the stability of peripheral deletional mechanisms, a method that allows for continued peripheral deletion over time must be devised in order to contend with newly emerging all-reactive T cells from the host thymus. This may be achieved through periodic dosing with specific depletional agents or perhaps through the development of genetically engineered donor tissue. A second advantage is the fact that once chimerism is achieved, it would be expected to be stable over time. However, it must also be emphasized that a loss of the chimeric state, for whatever reason, would almost certainly result in the subsequent rejection of the donor graft. Third, an important consideration in clinical tolerance protocols is the compatibility of immunosuppressive drugs with mechanisms of tolerance. While some agents have proven to be antagonistic in certain peripheral approaches to tolerance, no currently used immunosuppressive agent has been shown to inhibit the establishment of mixed chimersim. A fourth critical advantage of chimerism strategies is the fact that the induction of tolerance can be reliably quantified. Evaluating the persistence of donor cells in peripheral blood lymphocytes provides a reliable surrogate marker for the ongoing central deletion of donor-reactive T cells in the host thymus. Conversely, in peripheral deletional mechanisms, the myriad of T cell specificities as part of the allo-immune repertoire render it virtually impossible to measure whether all potential alloreactive clones have been eliminated and whether they will remain absent over time. However, peripheral mechanisms that rely on regulation do not suffer from this disadvantage since regulatory T cells could be measured, either by phenotyping or functional evaluation. Finally, in islet transplantation specifically, strategies for mixed chimerism may have the additional advantage over other mechanisms of tolerance in preventing autoimmune recurrence by restoring self-tolerance through the bone marrow transplant.

Despite these advantages, perhaps the most significant drawback for strategies to induce mixed chimerism in the lack of non-toxic preconditioning regimens that allow for routine engraftment of completely mismatched donor bone marrow without the risk of graft versus host disease. In this regard, peripheral deletional strategies have a distinct advantage as they allow for donor-specific elimination of the T cell compartment without the broad immunoablation required for the establishment of mixed chimerism. However, with advances in new immunomodulating therapies, together with collaboration between the evolving fields of marrow and organ transplantation, non-myeloablative strategies for mismatched donor bone marrow engraftment are on the horizon. It is important to emphasize that while strategies to induce tolerance are generally referred to in terms of their underlying mechanisms, it is not likely that the induction of tolerance in the clinic will depend on one mechanism alone. For instance, while central deletion through mixed chimerism may serve as a robust approach for clinical tolerance, the establishment of the chimeric state may rely on peripheral deletional or non-deletional mechanisms to facilitate the process. As novel strategies are developed for potential clinical translation, those strategies that combine mechanisms of tolerance induction will likely demonstrate the most promise.

8.2 SINGLE DONOR GRAFTS

As islet transplantation moves forward, one of the first challenges is to reliably achieve insulin independence with single-donor grafts. Based on experience with islet autotransplantation after total pancreatectomy, a minimum of 300,000 islets are necessary to achieve insulin independence in 70% of recipients (1). This is in stark contrast to the 850,000 islets required in the Edmonton series of patients, suggesting that factors such as the presence of autoimmunity, diabetogenic immunosuppression, and brain death of the donor may have detrimental effects on islet engraftment and function. Recently, studies performed in a rat model demonstrated that brain death significantly reduces islet yield and viability, as well as islet functionality as evidenced by in vitro static incubation and by in vivo implantation into syngeneic recipients (2). Advances in procurement techniques from cadaveric donors and improvements with less toxic and more potent immunosuppression will progressively lead to lower islet requirements to achieve normoglycemia (3).

8.3 LIMITED DONOR SUPPLY

Even if single-donor islet transplantation becomes consistently successful, the tremendous shortfall in the number of cadaveric pancreata would drastically limit the number of diabetic patients that could benefit from this procedure. Therefore, finding alternate sources of islet cells or the development of islet cell surrogates are critically important challenges before islet transplantation can be broadly applied in the treatment of diabetes. Recently, the University of Pennsylvania have demonstrated successful reversal of diabetes with single-donor islet transplants using organs procured from non-heart beating donors (4). At the other end of the spectrum, living donation of a segmental pancreas graft may also be an attractive alternative source for islets. Initial experience in living donor segmental pancreas transplants at the University of Minnesota revealed an increased risk to the donor of procedural complications and impaired glucose tolerance; however, more careful selection of donors has essentially eliminated these risks (5-8). Based on this experience, segmental pancreas grafts could be procured laparoscopically from living donors and subsequently used for islet transplantation, instead of a segmental pancreas transplant, thereby reducing the surgical risks to the patient (9). However, ensuring that an adequate islet mass can be obtained from a segment of pancreas in order to secure insulin independence will be a significant challenge in bringing this strategy closer to the clinic.

Xenotransplantation is another area of tremendous potential as an unlimited source of islet cells. Recently, in a nonhuman primate model of porcine islet xenotransplantation, the Minnesota group achieved long-term islet function with persistent porcine C–peptide using an immunosuppressive regimen consisting of basiliximab, FTY720, everolimus, anti-CD40L mAb, and leflunomide (10). While potent immunosuppression has been demonstrated to overcome xenograft rejection, another approach has been the development of transgenic pigs expressing human complement-regulatory proteins to effectively surmount immune destructive pathways (11). While these reports have been encouraging, the ongoing requirement of heavy immunosuppression and concerns regarding zoonotic viral transmission are significant obstacles limiting the clinical applicability of this approach. In recent years, controversy in xenotransplantation has achieved a new height with the report by Valdes et al. regarding improved glucose homeostasis by co-transplantation of pig sertoli cells and neonatal porcine islets in diabetic children (12). While this preliminary report demonstrates great potential, replication of these results in primates and a larger cohort of children will be required before definitive conclusions can be drawn (13).

While additional sources of islet cells are being investigated, the development of islet surrogates that are insulin-producing and glucose-responsive would completely eliminate the problem of supply and demand. Research in the area of stem cells has demonstrated considerable promise in recent years based on evidence of pancreatic stem cell proliferation using neogenesis peptides such as INGAP, hepatocyte growth factor, epidermal growth factor and gastrin (14). The opportunity for trans-differentiation of ductal elements into insulin-producing cells also provides another exciting opportunity for beta cell expansion (15). Substantial progress has also been made in genetic engineering, such as the transformation of hepatocytes to secrete a single-chain insulin analogue (16), and the alteration of intestinal mucosal K-cells to secrete insulin in response to hyperglycemia (17). While these strategies seem promising, concerns regarding imprecise physiological glucose homeostasis, potential transmission of malignancy and cellular rejection all need to be addressed as these approaches are further developed.

8.4 IMMUNOSUPPRESSION

The avoidance of diabetogenic agents, while maintaining adequate potency to contend with both allograft rejection and autoimmune recurrence, is a matter of tremendous importance as less toxic and more specific drugs enter the clinical arena.

Recently, the steroid-free sirolimus and low-dose tacrolimus-based protocol in the Edmonton experience has been shown to be highly effective in patients undergoing islet after kidney transplantation (18). In addition, the Minnesota group have implemented novel immunosuppressive protocols in their recent series of islet recipients, which has resulted in an unprecedented level of insulin independence after single donor islet infusions (19). The first series of patients received inductive treatment with a T cell depleting antibody, hOKT3γ_1-Ala-Ala, while the second series of patients received thymoglobulin induction, an anti-TNF-receptor drug (etanercept), and maintenance immunosuppression with mycophenolate mofetil and sirolimus. While the rates of single-donor islet transplant success have been impressive, major biasing factors have included use of only perfect-grade, high body weight pancreas donors, coupled with selection of low body weight and insulin sensitive recipients.

As novel immunosuppressive strategies move forward towards clinical application, a critical issue that requires careful consideration is the compatibility of immunosuppressive drugs with mechanisms of tolerance. While calcineurin inhibitors have played a vital role in improving outcomes in clinical transplantation, there is considerable evidence to suggest that they may interfere with mechanisms that enable the induction of stable tolerance. For instance, the induction of regulatory mechanisms have been shown to be dependent on calcium signals, therefore, while calcineurin inhibitors may be beneficial in preventing allograft rejection by inhibiting T cell proliferation, they may also be preventing long-term allograft acceptance by inhibiting the generation of dedicated regulatory T cells (20, 21). Moreover, calcineurin inhibitors prevent the secretion of IL-2, which is essential for T cell deletion via activation induced cell death. Therefore, by preventing IL-2 mediated apoptosis of activated T-cells, calcineurin inhibitor therapy may abrogate tolerance pathways. This has been clearly demonstrated in costimulation blockade-based strategies of tolerance induction, where the combination of calcineurin inhibitors with costimulation blockade prevents long-term allograft acceptance (21-23). In contrast to calcineurin inhibitors, sirolimus does not inhibit IL-2 secretion. Therefore, it allows for IL-2 mediated T cell deletion while also effectively controlling T cell clonal expansion (24). Li and colleagues demonstrated that while calcineurin inhibitor therapy prevented stable tolerance induced by costimulatory blockade, sirolimus synergized with co-stimulation blockade to induce massive apoptosis of alloreactive T cells and produced stable skin allograft tolerance (23).

In addition to the evolution of tolerance-compatible immunosuppressive protocols, another area of active research towards the avoidance of immunosuppression is the encapsulation of islets in immunoprotective devices prior to implantation. Several immunoisolation systems have been extensively studied and have been shown to enhance survival of both allogeneic and xenogeneic islets (25). However, the clinical application of these devices has been impeded by many important concerns including: adequate access of the encapsulated islets to blood supply and oxygen for survival; triggering of non-specific foreign body reactions to the biomaterials resulting in their destruction; and graft loss from cytokine-mediated immunological responses. While the concept of protecting islets is enticing, developments in polymer biology are definitely required before this approach can be applied to patients. In addition to islet encapsulation, considerable progress has also been made other immunomodulation strategies such as the genetic manipulation of islets to induce local immunosuppression, and the development of transgenic islets that are protected from immune mediated attack. While these approaches may one day allow more widespread application of islet transplantation, they are presently limited to experimental investigation. Therefore, maintenance immunosuppression is thus likely to remain with us and be a critical component of therapy at least in the near future, and may in fact be adjunctive to many of the above strategies (Figure 8-1).

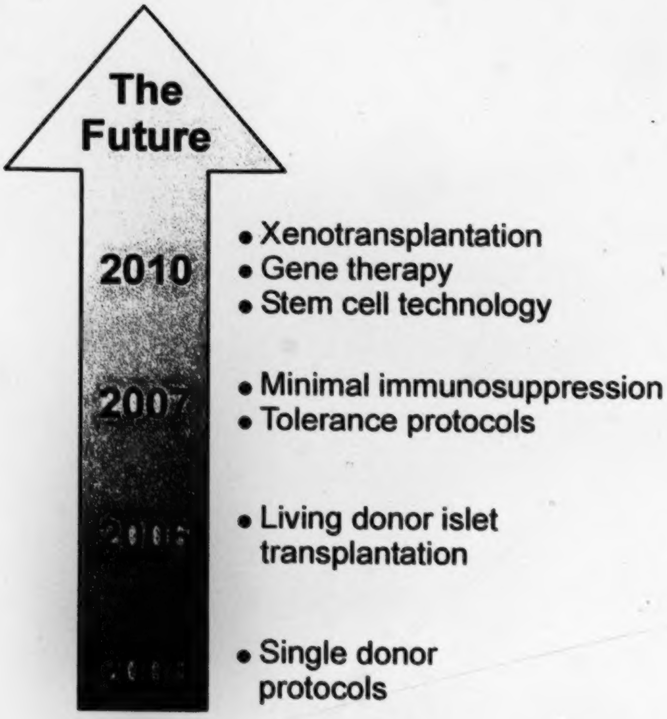
The Future
2010
• Xenotransplantation
• Gene therapy
• Stem cell technology
2007
• Minimal immunosuppression
• Tolerance protocols
• Living donor islet transplantation
• Single donor protocols

Figure 8-1: Future milestones in clinical islet transplantation.

8.5 CONSIDERATIONS IN TOLERANCE INDUCTION

Although tolerance was first experimentally induced almost half a century ago, the application of this phenomenon to the clinic has been dramatically more challenging than was initially envisioned. Although several successful tolerance-inducing strategies have been developed in rodent models, the application of these protocols to preclinical primate models or to humans has been largely ineffective. One of the most significant barriers to the translation of these strategies is the stark difference in the immune history between mice and non-human primates. Inbred rodents are housed in clean, pathogen-free facilities and have an inherently naïve peripheral T cell repertoire. Conversely, nonhuman primates and human patients have had previous immunological exposures and hence have a larger proportion of memory T cells in their repertoire. The existence of T cell memory poses a considerable barrier in transplantation since these cells are considerably more resistant to tolerance than their naïve counterparts (26-28). The clinical significance of this concept has been validated in patients undergoing renal transplantation, where a higher pretransplant frequency of previously primed memory T cells correlated to an increased risk of developing acute rejection episodes (29). More recently, it has been shown that prior environmental exposures to viruses can represent a potent barrier to tolerance induction due to the generation of virally induced alloreactive memory T cells (28). Moreover while depletional strategies have been generally accepted as tolerance promoting, recent evidence suggests that homeostatic proliferation of the residual T cells after depletion may in fact generate functional memory T cells that are resistant to tolerance induction (30).

An additional problem in the translation of protocols from small to large animal models is the lack of appropriate agents or the intrinsic differences in the toxicities of these agents between rodents and primates. A protocol that is safe and effective in rodents may not demonstrate the same effects in larger animals. For example, although anti-CD40 Ligand mAb therapy was highly effective at preventing graft rejection and inducing tolerance in rodents and nonhuman primate models, clinical application of this agent (Hu5C8) resulted in unexpected thromboembolic complications, resulting in the early termination of these trial (31, 32). This example also illustrates the importance of safety testing of novel protocols before their evaluation in clinical trials. This is particularly critical in clinical islet transplantation where the risk-benefit ratio must take into account that the underlying diabetic condition is not immediately life-threatening and, therefore, may not justify testing new strategies in this population.

It has been argued that achieving tolerance in the clinic will depend largely on strategies to induce mixed chimerism through bone marrow transplantation, without toxic pre-conditioning of the recipient (33). Based on a mechanism of central clonal deletion, strategies at inducing mixed chimerism have reliably proven to be the most robust from of tolerance. Experimental models have consistently demonstrated fortitude against the most rigorous tests for transplantation tolerance, and there is no evidence that any presently used immunosuppressive agent is antagonistic to the establishment of mixed chimerism (34). Moreover, there are case reports of bone marrow transplant recipients with established donor chimerism that have been able to accept a renal transplant from the same donor without further immunosuppression (35-38). Nevertheless, serious concerns regarding toxicities with recipient pre-conditioning and the risk of graft-versus-host disease have been major barriers to routine clinical application of these strategies.

In islet transplantation, strategies in mixed chimerism may be of particular interest since it has the potential of preventing autoimmune recurrence by restoring self-tolerance through the bone marrow transplant, in addition to achieving permanent islet allograft acceptance. However, until less toxic pre-conditioning strategies are developed, it is not justifiable to impose the current risks associated with bone marrow transplantation in a patient whose disease is controlled with insulin therapy. With advances in novel immunosuppressive agents, non-myeloablative strategies are rapidly evolving and the potential for clinical application in islet transplantation is becoming more realistic. For example, Seung et al. have demonstrated that the combination of total body irradiation with bone marrow transplantation and two doses of anti-CD40L antibody was able to prevent recurrence of autoimmunity and induce indefinite islet allograft survival in overtly diabetic NOD mice (39). Moreover, through low dose irradiation and antilymphocyte serum alone, Li et al. have been able to induce mixed chimerism and demonstrate indefinite islet allograft survival (40). Using agents that block costimulation or deplete lymphocytes, as described above, clinical non-myeloablative strategies are in development, and trials of donor bone marrow infusion combined with solid organ or islet transplantation are rapidly evolving (41).

One of the most difficult issues to address in the clinical translation of tolerance protocols is determining when in fact an individual is rendered appropriately unresponsive to donor antigen such that immunosuppression can be completely withdrawn. Starzl and colleagues investigated the concept of weaning immmunosuppression in a cohort of 72 liver, kidney and pancreas transplant patients who received thymoglobulin induction followed by tacrolimus monotherapy (42). Remarkably, they were able to wean approximately 60% of recipients to just interval dosing of tacrolimus. Similarly, Tanaka and colleagues at the University of Kyoto reported successful weaning in over 60 children that received living donor liver transplants, first to interval tacrolimus dosing followed by complete withdrawal of immunosuppression (43, 44). The mechanisms responsible for tolerance induction in these individuals remain a matter of speculation and intense investigation. Although promising in this cohort, complete withdrawal of all immunosuppression in islet transplant recipients will only succeed if both alloimmune and autoimmune destructive pathways have been overcome by a successful tolerance protocol.

It has been suggested that islet transplantation could serve as a primary test bed for novel tolerance protocols since failure to achieve tolerance would result in the patient's return to insulin therapy rather than potential death in the case of losing a life-sustaining heart or liver transplant. In addition, the fact that islets can remain in culture provides a unique opportunity not only to immununologically manipulate the graft, but also to optimally condition the recipient prior to transplantation. These advantages have generated tremendous interest to explore innovative tolerance strategies in islet transplantation. This enthusiasm is heightened by the growing number of anti-rejection therapies, either in the preclinical development or in early clinical trials that have demonstrated considerable potential in promoting tolerance. With these ongoing advances, the leap between experimental tolerance induction in the laboratory to the drug-free maintenance of normal allograft function in a transplant patient may not be too far off.

8.6 CONCLUSIONS

Although the concept of transplanting islets for the treatment of diabetes has existed for over a century, several technical and biological barriers have impeded clinical application of this approach in the past. However, in recent years, landmark advances in islet isolation and less diabetogenic immunosuppression have moved islet transplantation forward from research to clinical reality. With the introduction of the Edmonton Protocol and ongoing developments, islet transplantation has now been accepted as a safe and effective therapy for select patients with type 1 diabetes. At present, since patients receiving islet allografts must exchange insulin for lifelong immunosuppressive therapy, the procedure can only be justified in patients with very unstable forms of diabetes. Development of novel immunosuppressive protocols using more specific and less toxic drugs, ultimately towards inducing tolerance, is an important step in applying islet transplantation earlier in the course of the disease, including transplantation in children. Moreover, advances in identifying other sources of islet cells, together with progress in better understanding the biology of diabetes, will help increase the limited supply of islets through gene therapy, stem cell biology techniques or xenotransplantation. It is anticipated that continued international collaboration will further stimulate excitement in the field as innovative solutions are created to meet the remarkable challenges that lie ahead.

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